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DIPLOMARBEIT

Titel der Diplomarbeit

„Development of a Triplex Assay for the simultaneous detection of Trichothecene and Fumonisin producing *Fusarium* strains by real-time PCR“

Verfasserin

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Abbreviations

AFLP: Amplified fragment length polymorphism
AFTOL: Assembling the Fungal Tree of Life
BHQ: Black hole quencher
Bp: Base pair
C_T: Threshold cycle
CTAB: Cetyl trimethylammonium bromide
D3G: DON-3-glucoside
DEPC: Diethylpyrocarbonat
DGGE: Denaturating gradient gel electrophoresis
DNA: Deoxyribonucleic acid
dNTP: Deoxyribonucleotide triphosphate
DON: Deoxynivalenol
EC: European Commission
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme linked immunosorbent assays
ESI: Electrospray ionization
EtOH: Ethanol
FAM: 6-carboxyfluorescein
FHB: *Fusarium* head blight
FRET: Fluorescence resonance energy transfer
Fum1: Fumonisin gene cluster
HPLC: High performance liquid chromatography
ITS: Internal transcribed spacer
Kb: Kilo base pairs
LNA: Locked nucleic acid
MS/MS: Tandem mass spectrometry
NIV: Nivalenol
NOEL: No observed effect level
PCR: Polymerase chain reaction
PDA: Potato dextrose agar
PDB: Potato dextrose broth
poly(dIdC): Poly deoxyinosine deoxycystodine
PVP: Polyvinylpyrrolidone
qPCR: Quantitative polymerase chain reaction
QTL: Quantitative trait locus
RFLP: Restriction fragment length polymorphism
RT: Room temperature
SD: Standard deviation
Ta: Primer annealing temperature
TAE: Tris Acetic acid EDTA buffer
TDI: Tolerable daily intake
Te: Extension temperature
TE buffer: Tris-EDTA buffer
TEF-1α: Elongation factor 1α
TLC: Thin layer chromatography
Tm: Melting temperature
Tri5: Trichothecene gene cluster
Tris: 2-Amino-2-hydroxymethyl-propane-1,3-diol
ZON: Zearalenone

1. Introduction

1.1. Biological background

Grains are cultivated grasses (members of the monocotyledon family *Poaceae*) with fruit seeds used as staple diet for humans and animals. These cultivated grasses are composed of a starch-rich and less protein containing endosperm, the fat-rich cereal germ, the coadunate testa and pericarp and the protein-rich aleuron layer [1, 2]. Grains contain a high amount of starch, relevant amounts of proteins and only a low amount of fat. Moreover, they contain significant amounts of B vitamins like thiamine, riboflavin and niacin whereas only small amounts of biotin, folic acid and pantothenic acid. Vitamin A is only observed in maize and they contain no vitamin C [2].

1.1.1. Maize (zea mays L.)

Maize belongs to the family of *Poaceae* and has been domesticated by indigenous people since prehistoric times [3, 4]. Oldest maize relicts, which were found in a vale of South-Mexico are dated back to the 4th- 5th century B.C. [4]. Maize was distributed in the 15th century from Gaspé in Canada to Chile in South America [5]. After Spanish invasion in late 15th and early 16th century, "mahiz" as it is called originally from Caribbean people, was spread from Europe across Balkan states and Russia to India and China. In fact, since World War II maize is worldwide distributed as it is able to grow in diverse climates [4].
Since now, it was found any wild form of maize. Closest related plants are teosinte, tripsacum species and *Euchlaena mexicana* Schrad., Maize derives presumably from mutated teosinte [4]. Maize has a diploid set of 10 primary chromosomes (2n=20) called A-chromosomes which can vary in their centromer position, length and heterochromatic regions. Knobs are thereby non-centromeric chromosome regions consisting of heterochromatin [6], highly repetitive regions that were used by Barbara McClintock during the 1940ies and 1950ies to demonstrate the existence of transposable elements [7].

1.1.2. The genus *Fusarium*

Taxonomy

The need for a reliable taxonomic system originates from the time when *Fusarium* strains were identified as plant pathogens. Modern *Fusarium* taxonomy is based on the work of Wollenweber and Reinking [8] conducted in 1935 in the publication "Die *Fusarien*" [8]. Based on special characteristics, the authors reduced in this publication the quantity of *Fusarium* species to the manageable number of 142 [8].

Characteristics for the classification of *Fusarium*

Fusarium strains can produce several types of spores: macroconidia, microconidia and chlamydospores but not all *Fusarium* species have the ability to produce each spore type. Macroconidia are produced in a

stroma called sporodochium whereas microconidia are produced by the aerial mycelium. The third spore type, chlamydospores are thick-walled spores filled with a lipid like substance. The morphological shape of macroconidia and the presence or absence of microconidia and chlamydospores are relevant for the taxonomic classification. Moreover, morphological data, such as the growth rate and the pigmentation of colonies, are used to classify a subdivision of variant *Fusarium* species. Pigmentation, morphology and growth rate are however not a suitable criteria for characterization since the pigmentation varies in carbohydrate-rich media [9]. To overcome the drawbacks of classical species determination, molecular techniques and proteomics are additionally used to improve the reliability of classifications. Special genetic regions, namely ITS-1 and ITS-2 (internal transcribed spacer) have been demonstrated to be a good target for the taxonomic classification as their sequences are fairly specific and comprehensive sequence information is accessible via internet databases for numerous fungi (e.g. see the Fungal Tree of Life Project AFTOL http://www.aftol.org/index.php). Beside genetic approaches, also proteomics found its way into fungal phylogenetic classification. Proteomics is a technique to investigate the proteins at defined terms and conditions [10]. The most appropriate technique therefore is MALDI-TOF (Matrix-assisted laser desorption/ionization-time of flight mass spectrometry) that gives rapid and reliable results [11].

General characteristics of *Fusarium* Species

The genus *Fusarium* is associated with many plant diseases as these pathogenic fungi infect various cereal grains [12, 13]. Filamentous fungi of the genus *Fusarium* belong to the phylum Ascomycota, the largest division in the kingdom of the fungi. *Fusarium* is worldwide distributed in substrates like soil, plants and dead organic matter [9]. The most common teleomorphic state is *Gibberella*, which includes the species causing the highest economic damage like *Gibberella zae* (anamorph: *Fusarium graminearum*) and *Gibberella moniliformis* (anamorph: *Fusarium verticillioides*) [13]. Ascomycota can reproduce in a sexual or an asexual way, although the asexual form is more common. The asexual fertility concept- in which mitospores generate conidiospores (see figure 1), is the reason for the fast colonization of ecologic niches in barely tapped regions [7]. Frequent sexual fertility concepts are the heterothallic and the homothallic forms. Whereas the homothallic reproduction is a form of self-fertilization, the heterothallic reproduction form acquires a genetically different partner [13]. The first step in the life cycle of Ascomycota is the production of gametes in a specialized compartment named antheridium. Male gametes undergo repeated division after they migrated through thin hyphae into the ascogonium. The ascocarp, where karyogamy, meiosis and mitosis proceed, is formed by hyphae that contain two gametes. Products of the sexual reproduction are eight ascospores that are released upon appropriate circumstances [7].

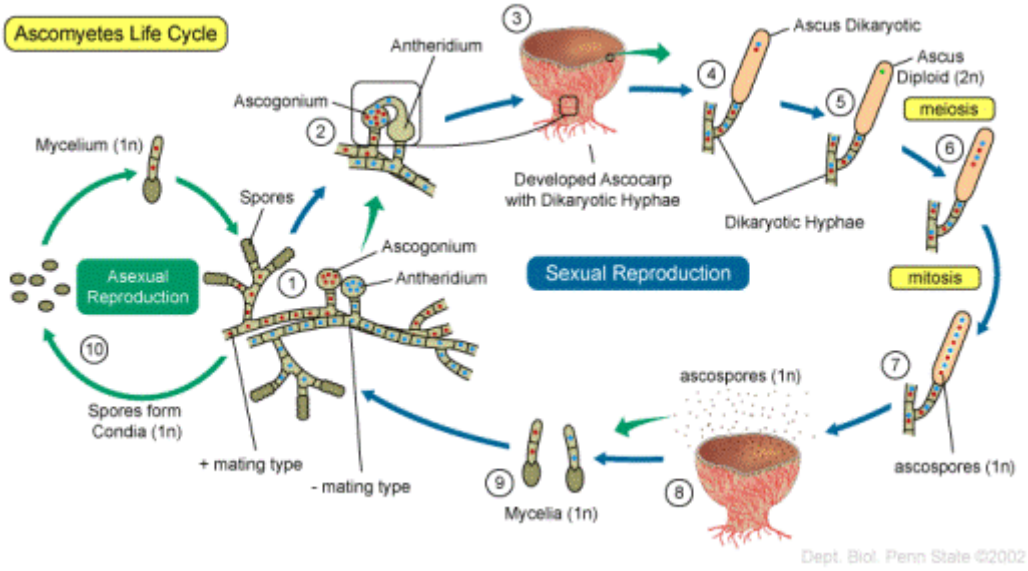


Figure 1 [14]: Life cycle of ascomycetes. Gametes are produced in the antheridium (male gametes) and the ascogonium (female gametes) (1). Male gametes migrate through thin hyphae into the ascogonium where they arrange in pairs and undergo repeated division (2). Hyphae with two gametes inside form the ascocarp where the karyogamy, the meiosis and following mitosis proceed (3-6). Finally, eight ascospores that are released by appropriate circumstances are the result of sexual reproduction (8). Asexual reproduction is more common. Condiospores that are dispersed in the environment are thereby produced by mitosis (10).

- ***Fusarium verticillioides* Nirenberg**

Fusarium verticillioides, former known as *Fusarium moniliforme*, is worldwide predominantly found on maize [13, 15]. Cultivated on Potato Dextrose Agar (PDA), the fungus initially produces white mycelium and develops violet pigmentation gradually, whereupon this pigmentation can reach from violet or dark magenta via orange to nearly black. *F. verticillioides* produces macroconidia that are characteristic for the *Gibberella fujikuroi* species complex *F. verticillioides* belongs to. Macroconidia produced by *F. verticillioides* are thin-walled, long and slender. Microconidia are abundant in the aerial mycelium and oval to club shaped. *F. verticillioides* does not produce Chlamydospores [13].

F. verticillioides causes cob rot, stalk rot and root rot resulting in reduced grain quality and yield loss on maize plants [13]. The fungus can infect maize by entering from the seed [16] or through wounds in the plant material [13].

It is suggested that maize varieties which are resistant to *Aspergillus flavus* infection are also more resistant to *Fusarium verticillioides* infection [17]. *F. verticillioides* can degrade 6-methoxy-2-benzoxazolinone and 2-benzoxazolinone, antimicrobial substances produced by maize which presumably is the reason for the success of this fungus as maize pathogen [18-23].

- ***Fusarium proliferatum*** **Nirenberg**

Fusarium proliferatum is worldwide distributed and causes several diseases on maize, sorghum, mango and asparagus. This fungus produces violet to black pigmentation on PDA media. Macroconidia are typically thin-walled, slender and relatively straight. Microconidia are club shaped and chlamydospores are not produced [13].

- ***Fusarium nygamai*** **Burgess & Trimboli**

Fusarium nygamai is abundant in hot, arid or also tropical wet areas only and it is associated frequently with sorghum, maize, cotton, native grasses, rice and soil. *F. nygamai* was observed in Australia (New South Wales), Malaysia, Indonesia and Sudan causing several diseases like root rots in broad beans (*Vicia faba*). Most colonies show a grayish-orange spore mass in the center when grown on PDA media. Macroconidia are slender, thin-walled and hyaline whereas microconidia are small, oval or club shaped. Chlamydospores are present in hyphae on the surface of media or in aerial mycelium and are hyaline to pale yellow colored [13].

- ***Fusarium graminearum*** **Schwabe**

Fusarium graminearum is associated with maize, wheat, barley and is distributed worldwide. Colonies grow rapid with a dense, orange to yellow colored mycelium. Macroconidia are slender and thick-walled and there are no microconidia produced whereas chlamydospores are usually found in the mycelium [13]. *F. graminearum* has the ability to degrade the antimicrobial substances benzoxazinoids, 6-methoxy-2-benzoxazolinone and 2-benzoxazolinone which are produced by maize plants [20]. *Fusarium graminearum* strains are associated with three important mycotoxins: Zearalenone (ZON), Nivalenol (NIV) and Deoxynivalenol (DON) [13].

Toxigenicity and Impacts

Fusarium species release remarkable amounts of toxic secondary metabolites which cause not only damages in plants, but also cancer and other growth defects in both humans and animals [13]. The produced mycotoxins in combination with reduced yield in infected fields make *Fusarium* spp. the economically most relevant fungal plant pathogen worldwide. Recent impacts regard especially wheat and barley farmers in the Midwest of the United States where *Fusarium* head blight (FHB) periodically causes deficits of several billion dollars [24]. *Fusarium* head blight, also called ear blight or scab, is associated with the following strains: *F. graminearum, Fusarium culmorum, Fusarium poae, Fusarium avenaceum and Fusarium sporotrichoides*. FHB affects mainly wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). Nevertheless, FHB results in a reduced seed quality and a low crop yield. The plants are predominantly infected by *F. graminearum*, whereas the other species are also frequent associated with the disease. All fungi causing FHB

are adapted to various environmental requirements e.g. *F. graminearum* grows up to 30°C and so it is found in warmer regions, while *F. poae* prefers temperatures around 20°C [25]. *Fusarium* head blight is obviously a disease that causes enormous crop failures and according to this high economic impacts. The losses in the US because of FHB were estimated at $ 2.7 billion for the period from 1998-2000 [26].

Resistance to *Fusarium*

Conidiospores of *Fusarium* sp. are widely distributed by wind and then settled on soil. They can rest for a long period in soil or grow on dead plant material until conidiospores or ascospores infect leafs and ears of a new plant generation. Ascospores are usually dispersed over a short distance only, explaining the enriched contamination of soil in monocultures by *Fusarium* sp. Conidiospores are dispersed mainly by wind and can overwinter on soil. In the second year, spores enrich in soil and contamination increases by dispersal such as in rainsplash. Both, wheat and maize are favorite host plants for *Fusarium* species hence allowing an annual infestation if no crop rotation with non-host plants or catch-cropsis is performed. Residues of the harvest are an excellent place for *Fusarium* to overwinter; therefore it is necessary to eliminate them or to till the dead organic matter into the field [27]. In a study of Dill-Macky [28] was shown the effect of wheat following crops and different types of tillage. The highest FHB incidence was observed when wheat followed maize, the lowest incidence when wheat followed soybeans. The infection grade was also lower in plowed fields as in no-till treated fields. The DON level was app. 25% lower in wheat following soybeans as wheat following wheat and 50% lower than in wheat following maize [28]. The planting date is most likely important for the mycotoxin contamination. It was shown for maize that an earlier planting date results in a lower contamination; but this positive effect can be undone by annual weather changes indeed. However, the maximum contamination is observed when crop flowering occurs at the same time as the spore release [29].

Resistance breeding

Plant breeding is considered as an effective method to reduce *Fusarium* infection and therefore minimizing mycotoxin contamination in food [30]. In recent studies, recombinant maize inbred lines were along with the resistant respectively susceptible parents artificially inoculated with the pathogen causing *Fusarium* ear rot. However, four QTL were detected on chromosomes 3, 4, 5 and 6 of which the QTL on chromosome 4 seems to be the QTL with the largest effect. This QTL could be a new locus for the resistance to *Fusarium* ear rot in new breeding programs [31].

Chemical resistance

Fungicides are usually synthetic organic compounds that differ in chemical structure and mode of action. Nevertheless, they are important to prevent poor yields and the resulting economical impacts, dearth and diseases in affected territories. Fungicides can be applied either directly on the plants though the adequate

time has to be found or with seed dressing where the chemical is applied before planting to protect the seeds. It can be distinguished between fungicides with local effect or protective fungicides and fungicides with systemic-xylem dispersal. Fungicides with local effects are applied on seedlings but also on adult maize plants. They do not integrate into the plant but rest on the surface. Main benefit of these fungicides is that they are compatible with plants and have a broad efficiency. However, they are washed out by rain and therefore must be applied again. Systemic fungicides like triazole block the ergosterol pathway in fungi and are therefore an efficient class of fungicides. Triazoles enter fast into stalk and leafs diffusing with systemic dispersal. However, application of fungicides in maize cultures is difficult due to the elevated plant height during the period of highest *Fusarium* susceptibility [32].

1.2. Mycotoxins

Mycotoxins are secondary metabolites with low molecular weight produced by filamentous fungi and they are harmful to both humans and animals [33]. The term mycotoxin has been used for the first time more generally after the turkey X disease in early 1960ies where app. 100,000 turkeys died due to fungal secondary metabolites named aflatoxins produced by *Aspergillus flavus* found in peanut meal [34].
These toxins enter the body either by contaminated foot, direct dermal contact or by the inhalation of spores [33]. Obviously it is the best to avoid contamination as mycotoxins are stable to high temperatures and resistant to chemical degradation [35]. The most relevant groups of mycotoxins are aflatoxins, trichothecenes, ochratoxins, fumonisins and zearalenones [10]. Mycotoxins can be detected by TLC, GC, HPLC, LC-MS/MS or immunological methods [10].

1.2.1. Fumonisins

Fumonisins are a group of hydrophilic mycotoxins which are produced by *Fusarium* ssp. that contaminate mainly maize crops worldwide [36-39]. Following *Fusarium* species are reported to produce fumonisins: *F. verticillioides* as main producer of fumonisin FB1*, F. proliferatum*, *F. nygamai*, *Fusarium anthophilum*, *Fusarium dlamini*, *Fusarium napiforme* and *Fusarium globosum* [for a comprehensive review see *[36]*]. *F. verticillioides* and *F. proliferatum* are the most frequent producers of fumonisins and can be recovered from maize kernels nevertheless they appear healthy [36].
Fumonisins can be divided into four groups: A, B, C and P though the B-group is the most important one and consists of four toxins FB1, FB2, FB3 and FB4, with FB1 as the most studied one [13, 40]. Fumonisins consist of a 20-carbon linear backbone with an amine at C2 and esterified tricarboxylic acids at C14 and C15 [40, 41].
Fumonisins are associated with human oesophageal cancer [42, 43] and neural tube defects in adults and infants when mothers consumed fumonisin contaminated maize products [44]. Moreover they are reported to cause leukoencephalomalacia in horses, pulmonary edema in swine, liver cancer in rats and neurodegeneration in mice [13, 36]. Fumonisins interfere with sphingolipid metabolism which causes

heavy intoxications in both humans and animals as they inhibit the enzyme ceramide synthase [45].
Long-term feeding studies demonstrated that purified fumonisin B1 causes both liver and kidney cancer in rodents. Actually, only 0.67 mg/kg body weight per day cause renal cancer in rats and 0.8 mg/kg body weight per day cause liver cancer in rodents. The no observed effect level (NOEL) is defined with 0.2 mg/kg body weight per day and the total maximum of tolerable daily intake (TDI) is fixed at 2 mg/kg body weight per day for FB1, FB2, FB3 alone or in combination in the European union [46, 47].

Biosynthesis of fumonisins

Different genes found in the *fum* gene cluster are involved in the production of fumonisins. The first step of the fumonisin biosynthetic pathway is catalyzed by a polyketide synthase which synthesizes the reaction from acetyl-CoA and eight malonyl-CoA to a polyketide. The polyketide synthase is encoded by the *fum1* gene. Further steps are catalyzed by transaminases, dehydrogenases, three cytochrome P450 monooxygenases, dioxygenases and acyl-CoA synthases leading to the final product FB1 (see figure 2) [41].

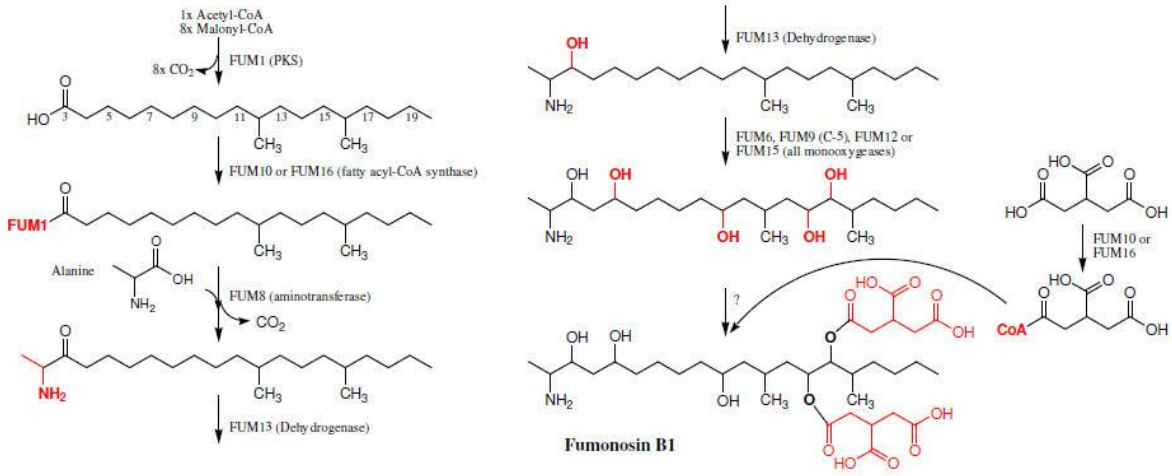


Figure 2 [modified from [41]]: Biosynthetic pathway for FB1 in *F. verticillioides*. 15 genes encoding for 15 enzymes are associated with fumonisin production [41, 48]. The *fum1* gene encodes for the polyketide synthase (PKS). PKS is the enzyme that catalyzes the first step from acetyl Co-A and eight malonyl-CoA to a polyketide. Alanine transaminase (Fum8) replaces the hydroxyl functional group with an amino group. Dehydrogenases (Fum7, *Fum1*3) add hydrogen to the double bond oxygen in C3. Fum6, *fum1*2 and *fum1*5 are genes encoding for cytochrome P450 monooxygenases. Fum3, former known as Fum9, is a dioxygenase which transfers oxygen to C5. *Fum1*0 is a fatty acyl-CoA synthase which appends CoA to tricarboxylates. *Fum1*1 is a tricarboxylate transporter. Tricarboxylates are transferred and added to the backbone [48].

Mode of action of fumonisins

Fumonisin B1 is a competitive inhibitor of ceramide synthase. Ceramides belong to the family of lipids and they are found in cell membranes where they can act as signal molecules. Nevertheless, the primary cause of the toxicity of FB1 is the accumulation of sphinganine and sphingosine which are cytotoxic substances with growth inhibitory effects (see figure 3). In addition to the accumulation of sphinganine

and sphingosine, the intake of FB1 promotes further apoptosis and mitosis which could play a role in tumor induction [49, 50].

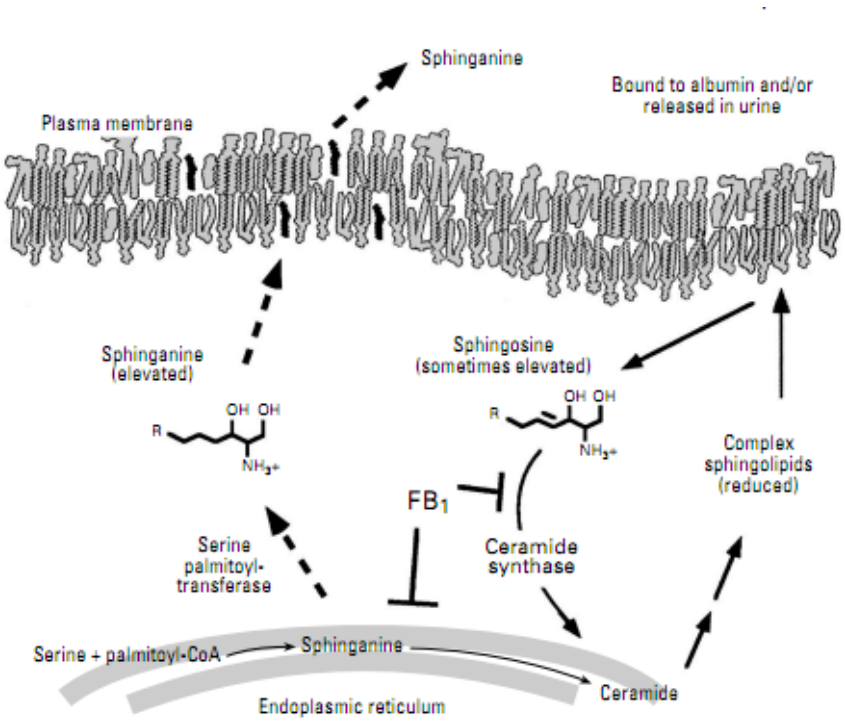


Figure 3 from [49]: FB1 occupies the place for sphinganine because residues of FB1 have structural similarities with sphingoid bases and fatty acyl-CoA in the enzyme ceramide synthase. This part can now interact with the binding site for sphingosine whereas the negatively charged groups can interact with the fatty acyl-CoA binding site [50]. However, when FB1 is present no ceramide can be produced from ceramide synthase from both sphingosine and sphinganine.

1.2.2. Trichothecenes

Trichothecenes are sesquiterpenoid worldwide occurring mycotoxins which are associated with *Fusarium* head blight [30]. Trichothecenes have central fused cyclohexene/tetrahydropyran rings with cyclopentyl residue adhered to the tetrahydropyran ring. Toxicity of trichothecenes derives from the epoxide group in C12 [51]. Functional groups, mainly hydroxyl or acetyl groups can vary in five positions (see figure 4). Trichothecenes are divided into four types A, B, C and D. *F. sporotrichiodies* and *F. poae* are producers of type A trichothecenes that include T-2 toxin and HT-2 toxin [52]. The most prevalent with FHB associated toxin is deoxynivalenol (DON) belonging to the type B trichothecenes mainly produced by *F. culmorum* and *F. graminearum* [52]. Type C and type D trichothecenes have a characteristic second epoxide group and are not associated with FHB [53].

Toxin	R1	R2	R3	R4	R5
DON	–OH	–H	–OH	–OH	=O
3-ADON	–OAc	–H	–OH	–OH	=O
15-ADON	–OH	–H	–OAc	–OH	=O
NIV	–OH	–OH	–OH	–OH	=O
T-2	–OH	–OAc	–OAc	–H	–OIsoval
HT-2	–OH	–OH	–OAc	–H	–OIsoval
4,15-DAS	–OH	–OAc	–OAc	–H	–H

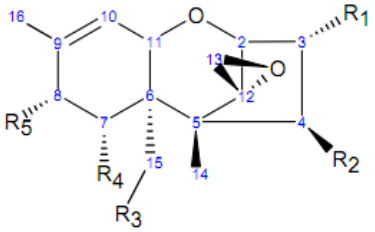


Figure 4 from [30]: Trichothecene structure.

Tri5 is the central gene of a 25kb gene cluster containing 12 co-regulated genes of which 10 genes are essential for trichothecene biosynthesis in the genome of *F. graminearum* [45].

As trichothecene toxins are harmful to the health of both humans and animals, organizations such as the European Commission set TDI maximum limits for the intake of DON (1 µg/kg body weight in unprocessed wheat, 200 µg/kg for infant food), ZON (0.2 µg/kg body weight) and a combined TDI for T-2 and HT-2 toxin (0.06 µg/kg body weight) [54].

Deoxynivalenol (DON)

Deoxynivalenol (DON) is a mycotoxin belonging to the group of trichothecenes, produced by *F. culmorum*, *F. graminearum* and other *Fusarium* species that contaminate grains [55]. DON is considered to cause nausea, vomiting and diarrhea in agricultural animals when ingested in large doses which gave DON its sobriquet vomitoxin. Nevertheless, DON is the most abundant trichothecene on maize, wheat, barley and rye [33].

Biosynthesis of trichothecenes

First, the initial substrate farnesyl-pyrophosphate is catalyzed by a trichodiene synthase (*Tri5*), a homodimeric enzyme with a 45kDa subunit [56] to trichodiene, a low-toxic product [51]. The *tri5* gene is only found in fungi that are able to produce type A and or type B trichothecenes [51]. Tri4, a multifunctional cytochrome P450 monooxygenase catalyzes the following four steps including epoxidation and hydroxylation reactions to isotrichotriol [57] and non-enzymatic isomerization to isotrichodermol [58], the skeleton of trichothecene structure which is finally acetylated by Tri101 [59] .

Mode of action of trichothecenes

Trichothecenes bind to the 60S ribosomal subunit thus inhibiting the translation of proteins and the further activation of a cell signaling pathway leading to apoptosis [60]. T-2 toxin leads to H_2O_2 production, inhibition of translation and to the stimulation of cell death in *Arabidopsis thaliana* at concentrations as low as 0.4 mg L^{-1} [61]. DON is reported to actually affect glial cells. In addition, it suppresses the uptake of the amino acid L-glutamate by astrocytes caused likely by the modification of the

expression of the transporter for glutamate at the cellular membrane [62].

1.2.3. Zearalenone (ZON)

Zeralenones (6-[10-hydroxy-6-oxo-trans-1-undecenyl]-B-resorcyclic acid lactones) are polyketides produced in the acetate-polymalonate pathway mainly in *F. graminearum*, *F. culmorum*, *F. equiseti* and *Fusarium crookwellense*. Zearalenone is reported to be a non-steroidal estrogen with significant estrogenic activity in animals leading to diseases such as hyperestrogenism in swine. Zearalenone compounds occur worldwide on maize, barley, oats, rice, rye, sorghum and wheat [63].

1.2.4. Detection methods of mycotoxins

Analytical detection of mycotoxins

Modern techniques to detect mycotoxin contamination or *Fusarium* infestation respectively on food are for instance analytical methods like LC-MS [64-66] or GC-MS [65]. Advantages of these methods are the low detection limits and the high accuracy. In mass spectrometer (HPLC/ESI-MS/MS) analysis a high quantity of mycotoxins up to 87 analytes can be measured after a single extraction step with acidified acetonitrile/water mixture [66]. In ELISA techniques, antibodies against *Fusarium* proteins have to be produced first. These antibodies bind to present antigens and can be detected colorimetric or by fluorescence [67].

Detection of fungal biomass

Fungal biomass can be detected by molecular methods like AFLP, RFLP [68] TEF-1α partial gene sequencing [16] and DGGE [68]. However, DNA based real time PCR assays to quantify *Fusarium* strains were introduced in the last years [12, 69-72].

1.3. The Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a method to amplify low amounts of DNA and was introduced by Mullis in 1986 [73, 74]. After seven years, Mullis was awarded the Nobel Prize in chemistry for his work on PCR [75].

1.3.1. General characteristics of the PCR amplification

PCR is a technique to amplify DNA billion fold within an hour. The exponential increase of DNA molecules is due to a three step cycle (see figure 5). The crucial component of the amplification reaction is an enzyme isolated from heat-stable bacterium *Thermus acquaticus* living in hot springs. This DNA-polymerase is in contrast to other enzymes heat-stable and can resist the high temperatures in PCR during the denaturation step of the reaction [76].

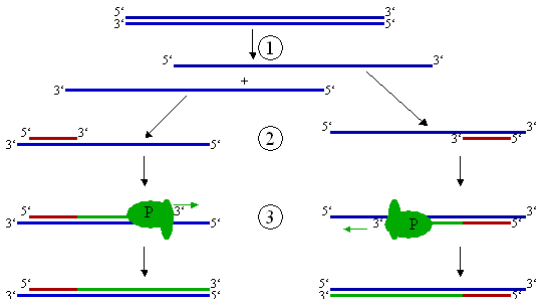


Figure 5 commons.wikimedia.org/wiki/File:Pcr.png: The steps of a PCR reaction. The amount of a target is exponentially increased (2^n). The required materials are low amounts of DNA, primers, nucleotides, buffer, $MgCl_2$ and a thermo stable DNA-polymerase.

However, PCR is not only impressive due to its high velocity but also because of its high specificity. Primers, particular oligonucleotides are complementary to the flanking regions of the target fragment. Primers define thereby the amplified region. It is crucial that primers are not complementary to each other in order that they do not form hairpin or stem-loop structures. There should be no complementarities in the 3' end of the primer to avoid false products and thereby a decrease of amplification efficiency [77]. The design of primers is usually performed *in silico* with specialized algorithms (e.g. primer3 plus [78]) to obtain the energetically most convenient sequence.

The development of PCR had a considerable influence on biological and biotechnological research. PCR has various fields of application; it was used to amplify DNA from 40,000 years old mammoth, it is used to amplify DNA from tissues or blood to investigate criminal acts as well as for human and veterinarian diagnostics. Due to this broad spectrum of applications a PCR thermocycler can now be considered as a basic equipment for all molecular biology and even for bio-analytical laboratories [7].

The initialization step:

Initialization is the first step of a PCR cycle. It is characterized by an extended heating of a few minutes to denature the DNA strands. This step is furthermore important to activate DNA polymerase in modern PCR kits which contain an "antibody-based hot start" polymerase [79].

The denaturation step:

After each cycle the DNA strands have to be denatured again. Typically, the temperature is set to 95°C to assure the breakup of hydrogen bonds between the nucleotide bases [7].

The primer annealing step:

At cooler temperatures, primers bind to complementary regions on single stranded DNA. The temperature of the annealing step depends on the length and sequence of the used primer pair. Primers with a high amount of adenine and thymine have a lower melting temperature as they form only double hydrogen bonds whereas guanine and cytosine form triple hydrogen bonds which melt at higher

temperature. The optimal primer annealing temperature is usually set 5°C below the melting temperature of the primers and can be calculated according to following equation:

$$Tm = 2\ °C\ \times (A + T) +\ 4\ °C\ \times (G + C)$$

The elongation step:

During the elongation step, the DNA-polymerase adds complementary nucleotides to the free 3' OH end of each primer. The optimal temperature is normally 72°C as this represents the temperature optimum for the *Taq* polymerase. However, for alternative polymerases other temperatures might be considered. The duration of this step depends both on type of polymerase and the length of the target sequence. If the whole reaction runs at the theoretical efficiency of 100% the DNA is doubled with each cycle [7].

1.3.2. Real-time Polymerase Chain Reaction (quantitative PCR)

The real-time PCR was developed in the 1996 and was introduced since then into various scientific fields to quantify nucleic acids. Real-time PCR assays are fast and easy to perform. Furthermore, they are robust and a later processing is not required. The quantification is done through real-time monitoring of the produced fluorescent signal [80]. DNA is detected by special intercalating dyes or by sequence specific fluorescent oligonucleotides. Although it is not important which detection system is used, the fluorescent signal is proportional to the amount of DNA [80].

Intercalating dyes

Intercalating dyes such as SYBR Green I, SYBR Gold, Eva-Green, ethidium bromide or YOYO I are sequence independent dyes, meaning that they bind to any present double stranded DNA. SYBR green I is an example for asymmetric cyanine dyes that binds to the minor groove of DNA. SYBR green is excited at a wavelength of 480nm and emits light at a wavelength-maximum of 520nm. A limiting factor of intercalating dyes is that they bind to all present DNA including PCR relicts like primer dimers or unspecific products. To prevent false positive signals a melting curve analysis of the product is frequently performed. In melting curve analysis the fluorescence signal decreases as a result of melting of double stranded PCR products. A single product must give a single peak, whereas multiple peaks indicate the formation of unspecific products or primer dimers. Moreover, the amount of product is direct proportional to the area under the peak [80].

Sequence specific probes

Sequence specific probes are used to increase the specificity of an assay by applying an additional oligonucleotide which is complementary to the product sequence. Due to the decrease of the quench of dual labeled probes the intensity of fluorescence signal can be related to the amount of formed DNA. As

the efficiency of quenching depends on the distance between the two fluorophors, the change in this distance is used to generate the sequence specific fluorescence signals [80].

Hydrolysis probes

Hydrolysis probes like TaqMan® probes are complementary to the target DNA sequence. These probes are 5' labeled with a reporter fluorophor and 3' terminally labeled with a quencher and normally, no fluorescence signal can be detected as the distance between quencher and fluorophor is short enough to allow a fluorescence energy transfer between the two molecules. During the primer extension step the 5' – 3' exonuclase activity of the DNA-polymerase cleaves the nucleotide backbone of the probe and releases free fluorophores. These fluorophores are now distant from their respective quenchers and the total fluorescence increases each cycle according to the formed products [80].

Molecular beacons

Molecular beacons are labeled terminally with quencher and reporter dyes but only the internal part of the oligonucleotide is complementary to the target DNA sequence. Terminal sequences are self-complementary to keep quencher and fluorophor close together. The thereby formed stem-loop can bind the DNA product during the primer annealing phase. The secondary structure opens and the quencher and the fluorophor are separated and the fluorescence increases [80].

1.3.3. Quantitative PCR analysis

In real-time PCR analysis, each curve consists of the initial lag phase, the log phase characterized by an exponential accumulation of PCR products and the third plateau phase. The cycle at which the signal curve intersects the threshold is called threshold value C_T (see figure 6).

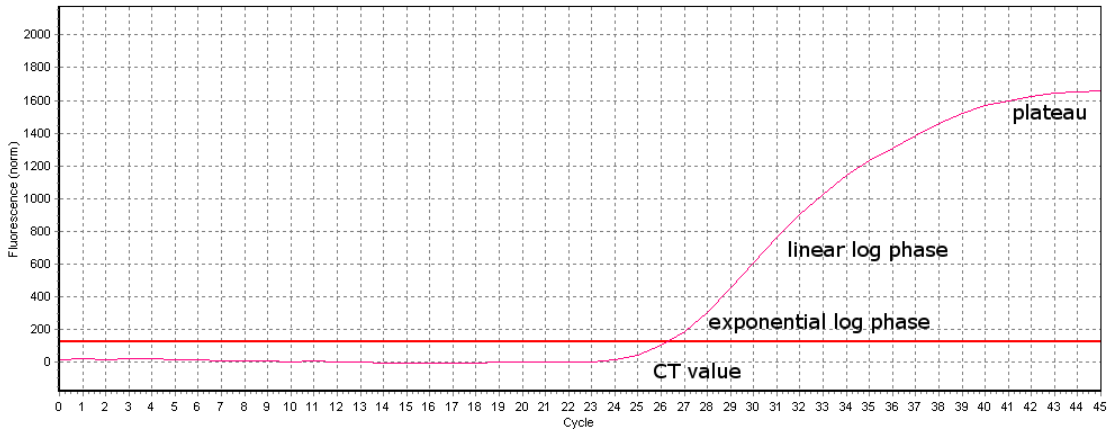


Figure 6: Real-time PCR curve. Initial lag phase, exponential log phase and plateau phase are shown.

For the quantification of DNA in a sample it is crucial to determine the efficiency of the assay. Furthermore, it is important that the efficiency of the standards is the same as for the unknown samples in the analysis. The efficiency can be calculated by following equation:

$$\varepsilon = 10^{-\frac{1}{slope}} - 1 \qquad 1.8 < \varepsilon < 2$$

Though real-time PCR assays show very high sensitivities, the knowledge of limit of detection (LOD) and limit of quantification (LOQ) determination is still poor. Recent studies of Nutz et al. [72] make use of the Youden index *J* to determine LOD and LOQ. The terms sensitivity and specificity are needed to calculate the Youden index. Thereby, sensitivity (Se) is defined as the fraction of known positive samples (standards) that appear positive in the assay. Specificity (Sp) is in contrary the fraction of known negative samples that appear negative. Both sensitivity and specificity are defined for each cycle to calculate the Youden index *J* [72] that is defined as: $J = Se + Sp - 1$ [81].

The cycle with the highest *J* value is thereby the optimal cutoff point. Additionally, LOD was determined as the amount of DNA that corresponds to the threshold cycle where 5% of the known positive samples appear negative. Additionally, the LOQ was determined as the ideal cutoff point in calibration curve. This cutoff point was first determined for each cycle as the threshold cycle with a maximum of specificity and sensitivity in sum [72].

The detection and quantification of *Fusarium* by PCR

Although molecular methods like AFPL, RFPL and DGGE are suitable techniques to detect *Fusarium* species qualitatively, real-time PCR analysis of samples is an ideal method for the quantification of strains [68]. Biosynthetic pathways and the corresponding genes were studies in order to design high specific primers to distinguish between mycotoxin producing and non-producing *Fusarium* strains [15, 70, 82-85]. With the knowledge of these sequences not only qualitative information can be gained by PCR but also quantitative information. Two different approaches, strain specific assays and group specific assays, have been developed for PCR detection of *Fusarium*. Strain specific PCR assays are based on sequence specific real-time PCR probes such as TaqMan® probes to detect single strains like *F. graminearum, F. culmorum* or *F. poae* which are main causers of FHB in Europe [86, 87]. Whereas in strain specific assays a diagnostic fragment for the detection of a single strain is analyzed, in group specific assays all strains which produce a special protein (e.g. an enzyme for mycotoxin biosynthesis) are detected simultaneously [12].

Several groups developed assays to detect and quantify fumonisin producing strains by using primers for the *fum1* gene encoding for a polyketide synthase [12, 68, 86, 88].

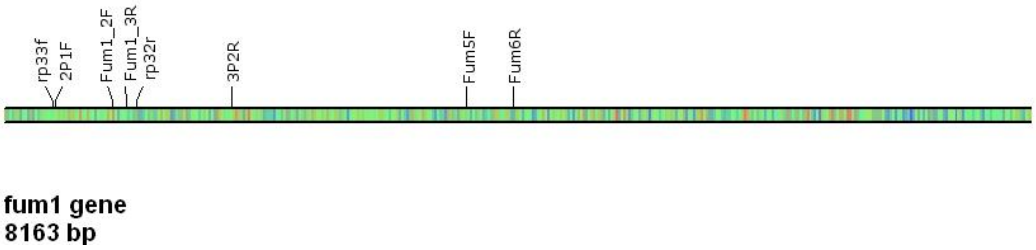


Figure 7: *Fum1* gene. Reported primers are indicated: rp33f and rp32r by Proctor et al; 2P1F and 3P2R by Nascimento da Silva et al.; *Fum1*_2F and *Fum1*_3R by Waalwijk et al.; Fum5F and Fum6R by Baird et al.

All trichothecene producing strains can be detected by primers targeting the *tri5* gene which encodes for the trichodiene synthase [68, 89-91]. The trichodiene synthase is the first enzyme of the biosynthetic pathway for the production of trichothecenes [92].

Multiplex PCR assays

Multiplex PCR assays are convenient methods to detect more than one target sequences in a single reaction only. These types of assays have many benefits as the number of separate PCR reactions can be reduced and thereby reagent consumption and analysis time decrease. To perform a multiplex PCR it is crucial to have a thermocycler which is able to excite and detect different fluorescent dyes. Moreover, the used dyes must differ in their emission spectrum to allow a separation of the formed products. For optimizations of multiplex assays it is important to minimize the primer concentrations to reduce primer-dimer formation and to increase the efficiency [93].

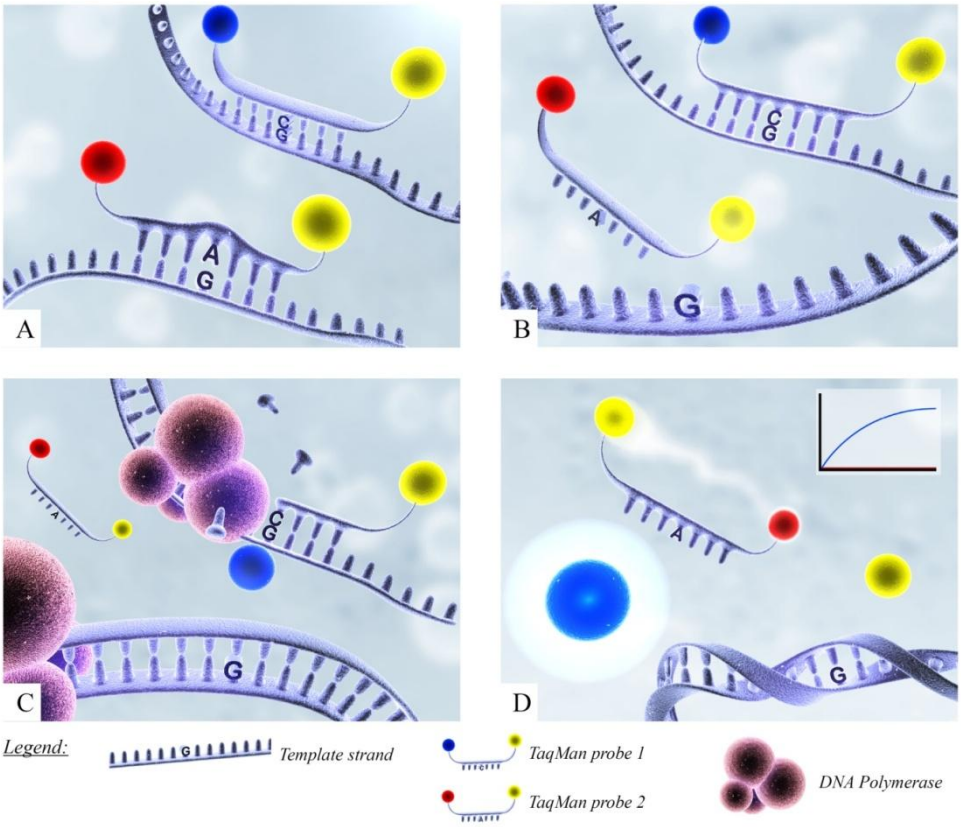


Figure 8 [94]: Two different TaqMan® probes are used for the detection of two DNA sequences. Each TaqMan® is labeled with a different fluorescent dye. A) The two probes bind sequence specific to the target sequence. B) TaqMan® probes just bind to the complementary sequence but not to other DNA fragments. C) DNA polymerase cleaves during the elongation step the probe. Fluorescent dye and quencher are separated. D) The separation of dye and quencher leads to the fluorescent signal.

1.3.4. Detection of fungal biomass by application of a reference gene system

In order to normalize the results obtained from different DNA extractions a reference gene system was introduced by Brunner et al. [91] using the example of the European Community Reference Laboratories (CRL) for genetically modified maize analysis [95]. This protocol refers the fungal DNA to the total extracted DNA from a sample. So the reference gene system can compensate for differences in the efficiency of DNA extraction [91]. The gene for an alcohol dehydrogenase in maize (*adh1*) was used to determine maize DNA.

$$inf.with\ fumonisin\ producing\ strains\ [\%] = \frac{fum1\ [ng]}{fum1\ [ng] + tri5\ [ng] + adh1\ [ng]} \times 100$$

$$inf.with\ trichothecene\ producing\ strains\ [\%] = \frac{tri5\ [ng]}{tri5\ [ng] + fum1\ [ng] + adh1\ [ng]}\ x\ 100$$

2. Aim of this study

The aim of this study is to combine three existing PCR assays to develop a triplex assay for the simultaneous detection of trichothecene and fumonisin producing *Fusarium* strains in a maize background:

Spadework carried out by the group of Dr. Kurt Brunner:

- A reference gene method was first developed for wheat samples in the diploma thesis of Maria Paula Kovalsky Paris [96]. As reference gene was used *ef-g1*, a translation elongation factor encoding gene in wheat.
- The reference gene method was adapted for maize. The gene for alcohol dehydrogenase in maize (*adh1*) was therefore used as reference gene.
- A duplex assay for the detection of *adh1* DNA and *tri5* DNA for trichothecene producing *Fusarium* strains was developed in the diploma thesis of Viktoria Preiser [97].

First of all, the duplex assay developed by V. Preiser will be applied to a comprehensive number of field samples. As the previous evaluation of this assay is based only on 20 samples, a high-throughput method will be used to study 240 samples obtained from a German partner, the "German maize committee, DMK". These samples include naturally infected maize obtained from eight different varieties cultivated on five different locations in Germany. A liquid handling station will be integrated into the assay set up to further enhance the throughput.

The development of a triplex real-time PCR assay will be carried out in two steps. First a duplex assay for the simultaneous quantification of the maize *adh1* gene and the *Fusarium fum1* gene will be designed. Thereafter, the *tri5* primers and probe will be included to end up with the desired triplex assay. This assay will then be evaluated carefully to guarantee the equal performance of the triplex assay as previously observed in the two duplex assays (*tri5*/*adh1* and *fum1*/*adh1*). Finally, the triplex assay will be used to study field samples with different levels of trichothecenes and fumonisins.

3. Materials and Methods

Following equipment was used in the experiments (see table 1):

Table 1: Used equipment

Equipment	Producer
Analytical balance, MC1	Sartorius, DE
Absorption photometry, NanoVue	GE life sciences, UK
Autoclave, Type 500, steam sterilizer	Varioklav, DE
Centrifuge, Centrifuge 5430	Eppendorf, DE
Centrifuge, GS-6 Centrifuge	Beckman, US
Centrifuge, GS-15 Centrifuge	Beckman, US
Centrifuge, Hetovac VR-1 Centrifuge w/Rotor	Heto Lab Equipment, DK
Centrifuge, Centrifuge 1-13	Sigma, DE
Centrifugation devices, Nanosep Omega 300kDa and 100kDa	Pall Corporation, US
Centrifuge tubes, sterile 15ml and 50ml	Greiner Bio-one, DE
Eppendorf tubes, 1.5ml and 2ml	Eppendorf, DE
Heater for centrifugation tubes	Grant Instruments, UK
Laminar flow, LaminAir® HB 2472	Heraeus instruments, DE
QIAgility	Qiagen, DE
pH meter	WTW, DE
Pipettor	Hirschmann Laborgeräte, DE
Pipettes, 10µl, 20µl, 100µl, 200µl, 1000µl	Eppendorf, DE
Power supply, Power Pac 300	Bio-Rad, DE
Real-time PCR 96 well plates and foils	Applied Biosystams, US
Shaking device, lab dancer digital	VWR, DE
Shaking device, MixMate	Eppendorf, DE
Shaking device, Thermomixer comfort	Eppendorf, DE
Thermocycler, 7500 Fast Real-Time PCR System	Applied Biosystems, US
Thermocycler, Mastercycler realplex² S	Eppendof, DE
Thermocycler, Rotor-Gene® Q	Qiagen, DE
UV-Transilluminator, Universal hood II – Gel Scan	Bio-Rad, DE
Vacuum pump, HetoVac VR-1	Heto Lab Equipment, DK
Water bath, GFL®	Müller-Scherr, Laborausrüstung, A

3.1. Fungal strains

Following strains were cultivated on potato dextrose (PDA) medium:
F. verticillioides, F. proliferatum 23*, F. proliferatum* 353*, F. proliferatum* 2763 and *F. nygamai.* These strains were cultivated and the extracted DNA was used as standards for real-time PCR quantification to determine the fumonisin producing *Fusarium* infection level on infected maize plants. Fungal strains were provided by the Institute of Biotechnology in Plant Production at IFA Tulln and the Institute for Chemical Engineering at Vienna University of Technology.

3.2. Maize cultivars

Artificially infected maize grown in Styria was obtained as ground samples from the Center for Analytical Chemistry at IFA Tulln.
Naturally infected maize samples were obtained milled from the German maize committee (DMK). Samples were obtained from five locations in all parts of Germany (see figure 9):

- Görzig, Saxony-Anhalt
- Ottmaring, Bavaria
- Lichtenau, Baden-Württemberg
- Prenzlau, Brandenburg
- Greven, North Rhine-Westphalia

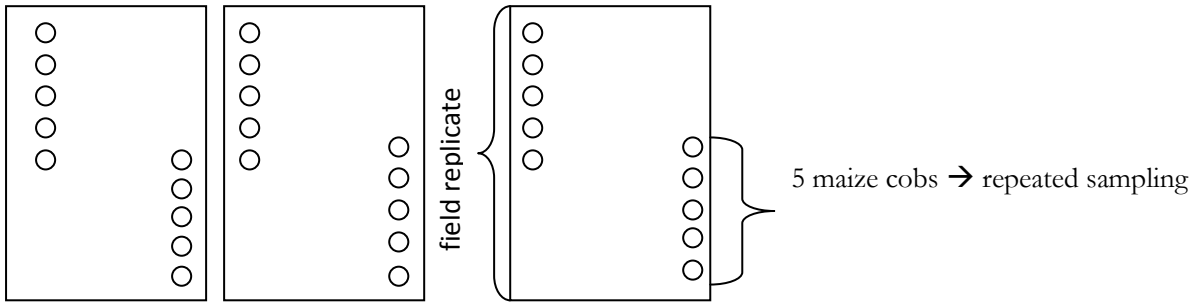


Figure 9: Setup of the field trials. Eight maize varieties were cultivated in three field replicates per location. Each field replicate was sampled twice by taking five cobs from subsequent plants.

3.3. Cultivation of *Fusarium* strains

3.3.1. Cultivation of fungal strains on Potato Dextrose Agar Medium (PDA)

For the preparation of PDA medium (Roth, DE), 39g of PDA were dissolved in 1000ml of deionized water. The solution was autoclaved, tempered to approximately 55°C and filled into Petri dishes. The fungal strains were inoculated on the Petri dish. *Fusarium* strains were cultivated on room temperature until the mycelium covered the whole Petri dish and were stored at 4°C for further needs.

3.3.2. The inoclulation of fungal strains in Potato Dextrose Broth (PDB)

24g PDB were dissolved in 1000ml of deionized water and then the solution was autoclaved. Flasks (250ml) containing 100ml of PDB were inoculated with a fungal mycelium obtained from freshly grown PDA plates and incubated on a rotary shaker at 150rpm and 30°C for 48h.

3.4. Molecular biology techniques

3.4.1. DNA extraction

Method A: CTAB extraction protocol

The CTAB extraction protocol was used for DNA extraction from maize kernels. This protocol is a modification of a protocol published by the Community Reference Laboratories (CRL) for extraction of maize DNA [98].

Extraction buffer: 1.4M NaCl (Merck, DE)
2% (w/v) CTAB (Roth, Germany)
0.1M Tris-Base (Merck, DE) (pH 8.0)
0.02M EDTA (Roth, DE) (pH 8.0)
1% (w/v) PVP 40000 (Roth, DE)

All reagents for the extraction buffer were dissolved in distilled water.

10% CTAB solution: 10 % (w/v) CTAB
0.7M NaCl

Reagents were filled up with distilled water.

Precipitation buffer: 1% (w/v) CTAB
0.05M Tris-Base (pH 8)
0.01M EDTA (pH 8)

Reagents were dissolved in distilled water.

Other reagents: Chloroform (Merck, DE): isoamyl alcohol (Roth, DE)(24:1)
70% EtOH (Baker Analyzed® Reagent, NL)
10mM Tris buffer

1g of fresh sample material was weighed into a 15ml flask and 6ml preheated CTAB extraction buffer (50°C) were added. During an incubation time of 1h at 70°C, tubes were mixed every 10 minutes briefly to ensure an increase DNA yield. After incubation, the tubes were cooled down and centrifuged for 15min at RT at 3,400rpm. After the centrifugation 500µl of the upper layer were transferred into a 2ml tube. 55µl of 10% CTAB solution and 550µl of a mixture of chloroform: isoamyl alcohol (24:1) was added and tubes were inverted 20 times. The samples were centrifuged at 7,200 x g for 5 min at RT. 350µl of the aqueous phase (upper layer) were transferred into a clean 1.5ml tube and 1,050µl precipitation buffer were added. The solution was mixed briefly and was kept for 15min at RT to allow precipitation of DNA. The samples were then centrifuged another time at 7,200 x g at RT for 15min. The supernatant was discarded and the pellet was was washed two times with 200µl of precooled 70% ethanol. Between the washing steps the samples were centrifuged 5min at 7,200 x g. The pellet was then vacuum dried and resuspended in 100µl 10mM Tris buffer (pH 8). Finally, the solution was heated in the thermo heater for 15min at 65°C to ensure that DNA dissolves in the buffer.

Method B: Large scale genomic DNA isolation protocol

Large scale genomic DNA isolation protocol is based on method of Saghai-Maroof at al [99] for green plant material. This extraction protocol was used to obtain maize leaf DNA which was later used as standard DNA.

Extraction buffer:	50mM EDTA
	700mM NaCl
	1% CTAB
	100mM Tris (pH 7.5) + 140mM beta-MercaptoEtOH (Sigma-Aldrich, DE)
Solution 1:	0.2M Na-OAc
	76% EtOH
Solution 2:	1M NH_4-OAc
	76% EtOH
TE buffer:	10mM Tris (pH 8.0)
	1mM EDTA

Dissolved in distilled water.

Other reagents:	Chloroform:isoamyl alcohol (24:1)
	Isopropanol

3 g of fresh sample material were homogenized by liquid nitrogen and were put into a clean 15ml flask. 9ml of CTAB extraction buffer preheated to 65°C were added. The samples were incubated for 1h at 65 °C and were mixed every 10 minutes briefly. Flasks were cooled down and 4.5ml chloroform: isooamyl alcohol (24:1) was added and the tube was shaken gently. Samples were then centrifuged for 10min at 1,500 x g at RT. The upper aqueous layer was transferred into a clean flask and other 4.5 ml of chloroform: isoamyl alcohol (24:1) was added. Samples were centrifuged 10 min at 1,500 x g again. The upper layer was transferred into a new tube and 6ml of isopropanol were added. The precipitated DNA was then collected with a glass hook and transferred into a 15ml flask containing 1ml TE buffer. 50 µl 5M NaCl and 2.5 ml 99% EtOH were added and the tubes were inverted. Precipitated DNA was first transferred with a glass hook into a new 1.5 ml tube containing 3 ml of solution 1 and immediately afterwards into a new tube containing solution 2 where the DNA was kept 20 min. Then the DNA was transferred into a clean 1.5ml tube containing 1ml TE buffer.

Method C: Phenol-chloroform extraction

Fungal DNA standards were obtained from the phenol-chloroform extraction.

CTAB solution: 1.4M NaCl
0.1M Tris
0.02M EDTA
2% CTAB

CTAB solution was set to pH 8.0 with HCl (Baker Analyzed® Reagent, NL), autoclaved and 2% beta-MercaptoEtOH were added.

Other reagents: Phenol (pH 7.9)
Chloroform: isoamyl alcohol (49:1)
Isopropanol
96% EtOH
10mM TE buffer (pH 8.0)

Fungal strains were cultivated in 100ml PDB for 48h. After incubation, mycelia were homogenized with liquid nitrogen and 600µl of a mixture of CTAB solution and 2% β-mercaptoethanol was added and kept for 15min at 65°C. 450µl phenol (pH 7.9) and 450µl of a mixture of chloroform: isoamyl alcohol (49:1) was pipetted into tubes containing the samples. Tubes were mixed gently until the solution appeared milky. Tubes were centrifuged at 13,000 x g for 5min at RT. Upper phase was transferred into a clean tube. Another 400µl of chloroform: isoamyl alcohol were added, centrifuged for 2min at 13,000 x g and the upper phase was then transferred into a clean tube. To precipitate the DNA, 0.7 volumes of

isopropanol were added and centrifuged for 20min at 13,000 x g at RT. Liquid was removed with a glass capillary and pellet was washed once with 500µl 96% EtOH. Tubes were centrifuged for 3min and the liquid was removed again. The pellet was dried under vacuum and was resuspended in 50µl TE buffer.

3.4.2. Determination of the DNA concentration and quality

The concentration of isolated DNA was measured by a NanoVue absorption photometer.
The sensor had to be wiped off first and then 2µl of nuclease free water or buffer were pipetted onto the sensor to obtain a blank value. Then 2 µl of the sample material were applied and measured. Absorption of DNA was measured at 260nm. However, the ratios between DNA and carbohydrates (260nm:230nm value) or DNA and proteins (260nm:280nm value) should be between 1.8 and 2.0 to ensure that the DNA is pure and no proteins or carbohydrates can later inhibit the PCR. The DNA was further diluted to a concentration of 100ng/µl with poly(dIdC) (Sigma, DE) and stored at -20°C to avoid enzymatic degradation.

3.4.3. Gel electrophoresis

TAE buffer (50x): 242g Tris
57.1ml pure acetic acid (Merck, DE)
100ml EDTA (0.5 M)
were filled up to 1000ml with distilled water.

Agarose (Serva, DE)

SYBR® safe 10,000x (Invitrogen, US)
Loading dye (6x) ready to use (Fermentas, CA)
FastRuler DNA Ladder, Low Range, ready to use (Fermentas, CA)

Depending on the desired agarose gel pore size the appropriate amount of agarose was mixed with 1x TAE buffer. The solution was heated up until the agarose was dissolved in the buffer. After cooling down to approximately 50°C, the appropriate volume of SYBR® safe was added and poured into a gel tray containing a comb. After polymerization, the agarose gel was transferred into the electrophoresis chamber. The chamber was filled with 1x TAE buffer and the comb was removed. The ladder and the sample DNA mixed with loading dye were placed into the slots. The electrophoresis was running with a voltage of 90V.

3.4.4. Real-time PCR

Kapa Probe Fast Universal 2X qPCR Master Mix (Peqlab, DE)
Kapa SYBR Fast qPCR Universal (Peqlab, DE)

Primers and probes (Sigma, DE):

All primers were ordered from Sigma-Aldrich and were delivered lyophilized. The primers were dissolved in 10mM Tris buffer (pH 8.0) to a final concentration of 100µM. Afterwards, the primers were diluted 16fold with water to reach a concentration of 100pM in the master mix.

PCR assay for the quantification of the *fum1* gene

Cultivated *Fusarium* strains were tested by the *fum1* assay modified from [88]. The amplified fragment has a length of 149bp. The sequences for the *fum1* primers are shown in table 2.

Table 2: Primers for the single detection of *fum1* gene

Primer		Sequence	Fragment length
***fum1* primer [88]**	forward	5' ATGCAAGAGGCGAGGCAA 3'	149bp
	reverse	5' GGCTCTCAGAGCTTGGCAT 3'	
***fum1* probe**	TaqMan®	5'[Cy5]CAATGCCATCTTCTTGAAACCT [BHQ1]3'	

The reaction volume was defined with 15µl containing 13µl master mix and 2µl DNA (see table 3). To avoid contaminations, filter tips and DEPC (Diethylpyrocarbonat) (Roth, DE) treated RNase free water were used. To compensate the loss while pipetting, an excess of 15% was calculated. The master mix was made in LoBind tubes (Eppendorf, DE) and was afterwards aliquoted with 10% excess. Each sample was measured as triplet. Accordingly, 42.24µl of master mix and 8µl DNA were mixed for one sample (see table 3). 15µl of this mixture were transferred into a 96 well plate (Eppendorf, DE and Applied Biosystems, US) and sealed with adhesive film (Applied Biosystems, US). The plate was then mixed for 30sec at 1,900 x g and centrifuged for 30sec at 2,000 x g.

Table 3: Pipetting schema for the production of the master mix

Substance	Volume per reaction [µl]
Kapa probe fast master mix	7.5
DEPC RNase free water	4.78
Forward primer	0.24
Reverse primer	0.24
TaqMan® Probe	0.24
Final volume of the master mix	**13**
DNA	2
Reaction volume	**15**

Primer sequences for the *fum1* singleplex assay were gained from Waalwijk et al. [88], the TaqMan® probe was modified. The appropriate temperature program was established by temperature gradient tests (52-62°C for the primer annealing step) with a DNA amount of 1ng/µl (see table 4 for the final temperature program).

Table 4: Final temperature program for the *fum1* assay

Step	Temperature	Time	Repeated
Initialization	95°C	1:50min	
Denaturation	95°C	15sec	45x
Primer annealing and elongation	58°C	45sec	45x

Clean-up of DNA used as real-time PCR standard

Fragmented DNA adulterates the C_T values and therewith the results of the PCR reaction. Several tests were made with various spin columns (NanoSep 300kDa and 100kDa). First, different sized DNA ladders were tested with different centrifugation times and several washing steps. NanoSep 100kDa devices were later used for the separation of fragmented DNA from the full length genomic DNA. 100µl of maize and wheat DNA respectively were centrifuged for 5min at 1000 x g. Then 200µl of TE buffer were added and the samples were centrifuged again at 1000 x g for 5 min. The samples were applied on an agarose gel and the results were confirmed by real-time PCR.

Development of a triplex assay for the simultaneous detection of *fum1*, *tri5* and *adh1* genes

Special reporter dyes are used for multiplex assays. These different reporters are used for the TaqMan® probes which are detected by the Applied Biosystems 7500 Fast Real-Time PCR System or Rotor-Gene® Q.

- The simultaneous quantification of *tri5* and *adh1* genes: The *tri5/adh1* duplex assay

The duplex assay for the simultaneous detection of maize and trichothecene producing *Fusarium* strains was developed in preceding studies accomplished by Viktoria Preiser (see table 5).
This assay was verified in the present work with a large set of maize samples.

Table 5: Temperature program for the *adh1/ tri5* duplex

Step	Temperature	Time	Repetition times
Initialization	95°C	1:50min	
Denaturation	95°C	15sec	45x
Primer annealing and elongation	59°C	45sec	45x

All *adh1* primers and the *tri5* probe were used 50pM, all other primers were used 100pM.
Maize DNA from leafs was used as standard in a concentration of 25ng/µl; as *Fusarium* standard was used DNA from *F. graminearum* in a concentration of 1ng/µl in a background of 25ng/µl maize DNA.

- **The simultaneous quantification of *fum1* and *tri5* gene: The *fum1*/*tri5* duplex assay**

Fum1 and *tri5* primers had to be tested for putative interactions first. Therefore, primer pairs were combined in a single master mix without DNA template. Primers were additionally tested with the wrong DNA: *fum1* primers were tested for amplification of *tri5* producing *Fusarium* strains and *tri5* primers were tested for *fum1* producing strains. Furthermore, several annealing temperatures and primer concentrations were tested. The *tri5* primer sequences are shown in table 6. The pipetting schema for a duplex assay is shown in table 7.

Table 6: *tri5* primers and probe for the *fum1*/ *tri5* duplex

Primer		Sequence	Fragment length
***tri5* primer [91]**	forward	5'GATTGAGCAGTACAACTTTGG 3'	178bp
	reverse	5' ACCATCCAGTTCTCCATCTG 3'	
***tri5* probe**	LNA-TaqMan®	5'[6FAM]C[+C][+T] [+T] [+G]G[+G]CCA [BHQ1]	

Table 7: Pipetting schema for the duplex assay

Substance	Volume per reaction [µl]
Kapa probe fast master mix	7.5
DEPC RNase free water	4.06
Forward primer *adh1*	0.24
Reverse primer *adh1*	0.24
TaqMan® Probe *adh1*	0.24
Forward primer *fum1*	0.24
Reverse primer *fum1*	0.24
TaqMan® Probe *fum1*	0.24
Final volume of the master mix	**13**
DNA	2
Reaction volume	**15**

Various tests were also made to determine the best standard mixture (see table 8):

Table 8: Different concentrations for the standard mixture

Maize DNA conc.	*Fum1* DNA conc.	*Tri5* DNA conc.	tenfold diluted with:
40ng/µl	1ng/µl	1ng/µl	Maize DNA
/	/	10ng/µl	p(dIdC)
/	10ng/µl	/	p(dIdC)
40ng/µl	1ng/µl	1ng/µl	p(dIdC)

Table 9: Final temperature program for the *fum1*/ *tri5* duplex assay

Step	Temperature	Time	Repetition times
Initialization	95°C	1:50min	
Denaturation	95°C	15sec	45x
Primer annealing and elongation	58°C	45sec	45x

- **The simultaneous quantification of *fum1* and *adh1* genes: the *fum1/adh1* duplex assay**

Primers of *fum1* and *adh1* assay had to be tested for putative interactions first with SYBR mix- but without DNA template; with the temperature program for *adh1* singleplex assay and a further melting curve experiment. Temperature program for *adh1* was a three step assay (see table 10):

Table 10: Temperature program for the *adh1* detection

Step	Temperature	Time	Repetition times
Initialization	95°C	1:50min	
Denaturation	95°C	15sec	45x
Primer annealing	58.6°C	15sec	45x
Elongation	66.3°C	20sec	45x

For each reaction, the whole *adh1* primer set was used but only one primer of the *fum1* primer set. The sequences for the *adh1* primer set are seen in table 11.

Table 11: *Adh1* primers

Primer		Sequence	Fragment length
***adh1* primer [CRL]**	forward	5'CGT CGTTTCCCATCTCTTCCTCC 3'	136bp
	reverse	5' CCACTCCGAGACCCTCAGTC 3'	
***adh1* probe**		5'[JOE™]AATCAGGGCTCATTTTCTC GCTCCCA [BHQ1]3'	

The temperature program for the *fum1/adh1* assay is an adaptation of both singleplex assays. To work towards the triplex assay, the temperature program was the same as for the *fum1/tri5* duplex assay (see table 12).

Table 12: Temperature program for the *adh1/fum1* duplex

Step	Temperature	Time	Repetition times
Initialization	95°C	1:50min	
Denaturation	95°C	15sec	45x
Primer annealing and elongation	58°C	45sec	45x

Maize DNA was spiked for the standard mixture with *fum1* DNA in the following concentrations:

- Maize: 45ng/µl
- *Fusarium* DNA: 5ng/µl

Standard DNA was three times tenfold diluted and each dilution was measured as triplet. Singleplex assays for *adh1* and *fum1* were performed additionally to ensure the same efficiency for the singleplex and the duplex assay.

- **The *tri5/fum1/adh1* triplex real-time PCR assay**

The primer pairs are the most sensitive part in the development of the triplex assay. If primers interact among themselves, the assay could either not work, or could give poor signals. For this reason, the primers were tested with template DNA of the opposite primers e.g. each *Fusarium* DNA was tested with *adh1* primers and the other way around.

The assay was optimized with different primer concentrations. As with a decreased primer concentration the potential interactions are reduced but also the fluorescence signals decreases, it is essential to evaluate these parameters. The *adh1* primers and the *tri5* probe were therefore set on 50mM and all other primers were used in the 100pM concentration.

4. Results

4.1. The *fum1* singleplex assay

The *fum1* singleplex assay was performed with *fum1* primers to determine if the primers work for different *Fusarium* strains with a similar efficiency (see table 13). DNA of five *Fusarium* isolates was extracted with the phenol-chloroform extraction protocol. For facility of inspection, is shown just a dilution series for one strain (*F. verticillioides*) in figure 10. The DNA was diluted fourfold from initial 1ng/µl. The distance between two fourfold dilutions should be two C_T values.

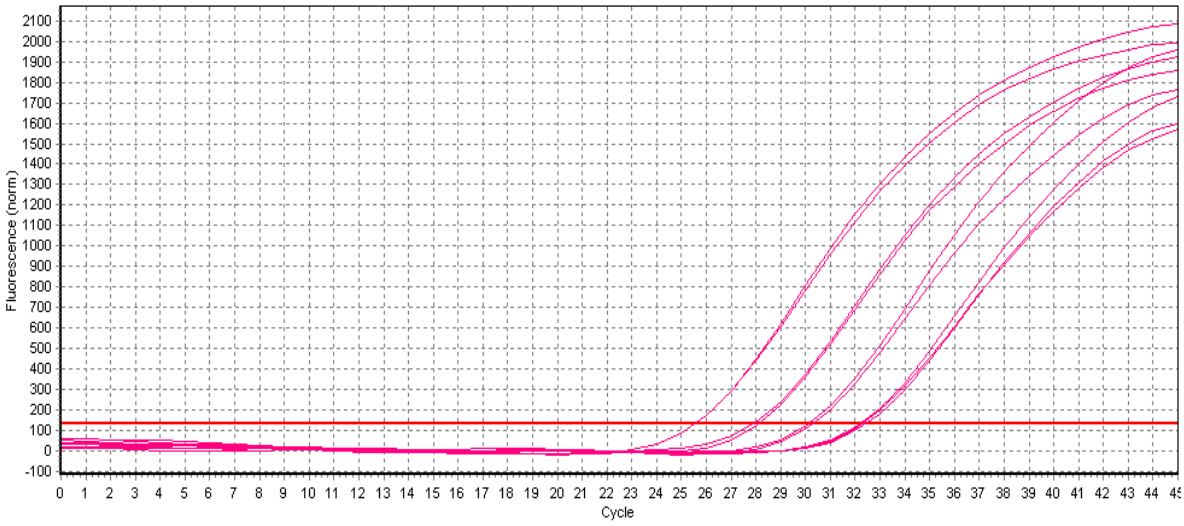


Figure 10: *F. verticillioides* DNA is shown in a fourfold dilution series. According to this dilution series, the efficiency of the assay was calculated.

Table 13: Each *Fusarium* isolate was measured in a dilution series to calculate the efficiencies

Strain	DNA concentration	Averaged CT value	Distance between CT values	Efficiency
***F. proliferatum* 23**	1ng/µl	27.1		0.85
	0.25ng/µl	29.5	2.4	
	0.0625ng/µl	32.0	2.5	
	0.0156ng/µl	33.8	1.8	
***F. proliferatum* 353**	1ng/µl	26.5		0.92
	0.25ng/µl	28.3	1.8	
	0.0625ng/µl	30.7	2.4	
	0.0156ng/µl	32.6	1.9	
***F. proliferatum* 2763**	1ng/µl	24.3		0.89

	0.25ng/µl	26.7	2.4		
	0.0625ng/µl	28.7	2.0		
	0.0156ng/µl	30.8	2.1		
F. verticillioides	1ng/µl	25.6			0.88
	0.25ng/µl	28.0	2.4		
	0.0625ng/µl	30.2	2.2		
	0.0156ng/µl	32.3	2.1		
F. nygamai	1ng/µl	27.8			0.87
	0.25ng/µl	30.2	2.4		
	0.0625ng/µl	32.5	2.3		
	0.0156ng/µl	34.5	2.0		

The efficiency of the singleplex assay can be calculated by means of a dilution series for each measured strain by

$$\varepsilon = 10^{-\frac{1}{slope}} - 1$$

4.2. The purification of standard DNA

The same concentration of DNA should give approximately the same C_T value if the PCR efficiency is identical. It is obvious from figure 11 that a wide range is between the single C_T values of isolated DNAs.

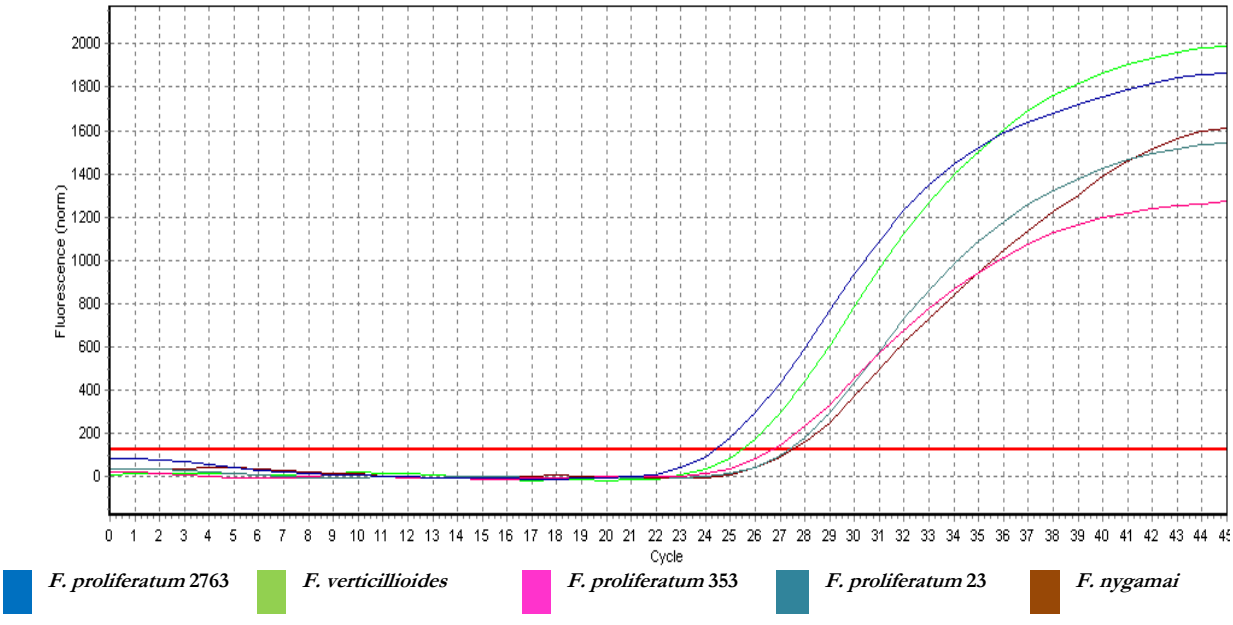


Figure 11: All strains were subjected to a real-time PCR in a 1ng/µl concentration. The C_T values of *F. verticillioides* and *F. proliferatum* 2763 are apparently lower than the other C_T values.

To proof if the differences result from fragmentation of the DNA or comes from a different amount of DNA, a 2% agarose gel was made (see figure 12). DNA ladder and loading dye were applied on the outer left and right side. 10µl of a 6ng/µl concentrated DNA were applied in each slot. It is obvious from figure 12 that the applied DNAs in slot i and slot v show less fragmentation than the DNAs in slots ii-iv. The more fragmented DNAs have bands on 50bp whereas the less fragmented DNAs consist mainly of genomic DNA.

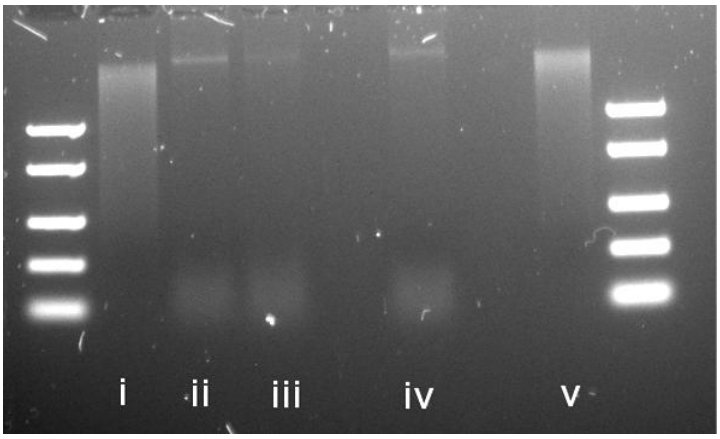


Figure 12: Fragmented *Fusarium* DNA is visible on the agarose gel. The marker is applied on the first and the last slot. i) *F. verticillioides* 16 ii) *F. nygamai* iii) *F. proliferatum* 23 iv) *F. proliferatum* 353 v) *F. proliferatum* 2763

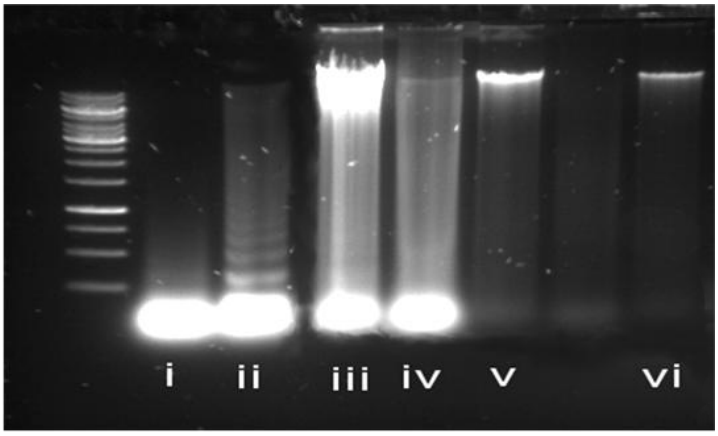


Figure 13: Purified DNA from *Fusarium*. The marker is applied into the first slot. i) Filtrate of DNA purified with 100kDa devices ii) Filtrate of DNA purified with 300kDa devices iii) Retentate purified with 100kDa devices iv) Retentate purified with 300kDa devices v) Retentate purified with 100kDa, washed once vi) Retentate purified with 100kDa, washed twice

Purified DNA samples without washing are shown in slots iii and iv (see figure 13). In slot v is the DNA with one further washing step and in slot vi with two washing steps. As it is illustrated in figure 13 the non-washed DNA has thick bands for genomic DNA but also thick bands for small fragments and a smear between the bands.

A real-time PCR was performed with the same amount of purified and the non-purified *Fusarium* DNA samples. The results from the real-time PCR are shown in table 14.

Table 14: Comparison of the C_T values before and after purification with 100kDa devices

Strain	C_T value of non-purified DNA	C_T value of purified DNA	Range between C_T values
***F. proliferatum* 23**	31.7	29.0	2.7
***F. proliferatum* 353**	34.5	28.9	5.6
***F. proliferatum* 2763**	25.6	25.7	-0.1
F. verticillioides	25.2	24.4	0.8
F. nygamai	34.0	29.2	4.8

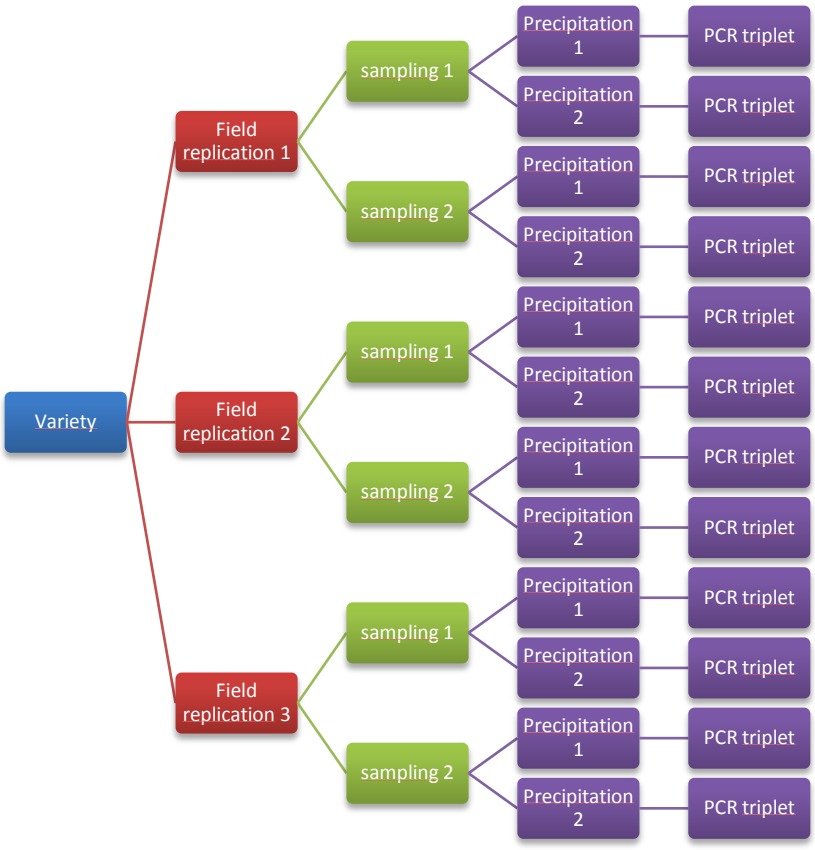
4.3. The duplex assay to quantify maize DNA and the DNA of trichothecene producers

The samples of the German maize committee were measured with the *adh1/tri5* duplex after they were extracted with the extraction protocol A (CTAB extraction protocol). 25ng/µl naturally infected maize sample was measured with the *adh1/tri5* duplex assay on the Applied Biosystems cycler.

Four twofold dilutions were used as maize standard and a mixture between genomic DNA of trichothecene producing isolates and maize DNA was used as *Fusarium* standard.

Starting concentration for the *Fusarium* standard was 1ng/µl *tri5* DNA in a background of 25ng/µl maize DNA which was diluted fourfold with 25ng/µl maize DNA for five times.

The experimental set-up for the analysis of maize varieties was performed according to schema 1:



Schema 1: Experimental set-up for the analysis of maize varieties

Table 15: Maize varieties used for the experiments

Abbreviation	Variety
PR	PR39F58
Zi	Zidane
LG 3220	LG 3220
NK	NK Nekta
LG 3258	LG 3258
Gr	Grosso
Ri	Ricardinio
Su	Susann

4.3.1. The comparison of the total infection grade on the different locations

Naturally infected maize samples were planted in five locations distributed all over Germany. Görzig is located in East Germany, Greven in Nord-West Germany; Ottmaring is located in South-Germany like Lichtenau which is found in South-West Germany. Prenzlau is located in North-East Germany.

The general level of infection over all used varieties is illustrated in figure 14. The infections reach from 0.4% for the lowest infection grade represented in Greven to a mean of 5.2% infection in Görzig.

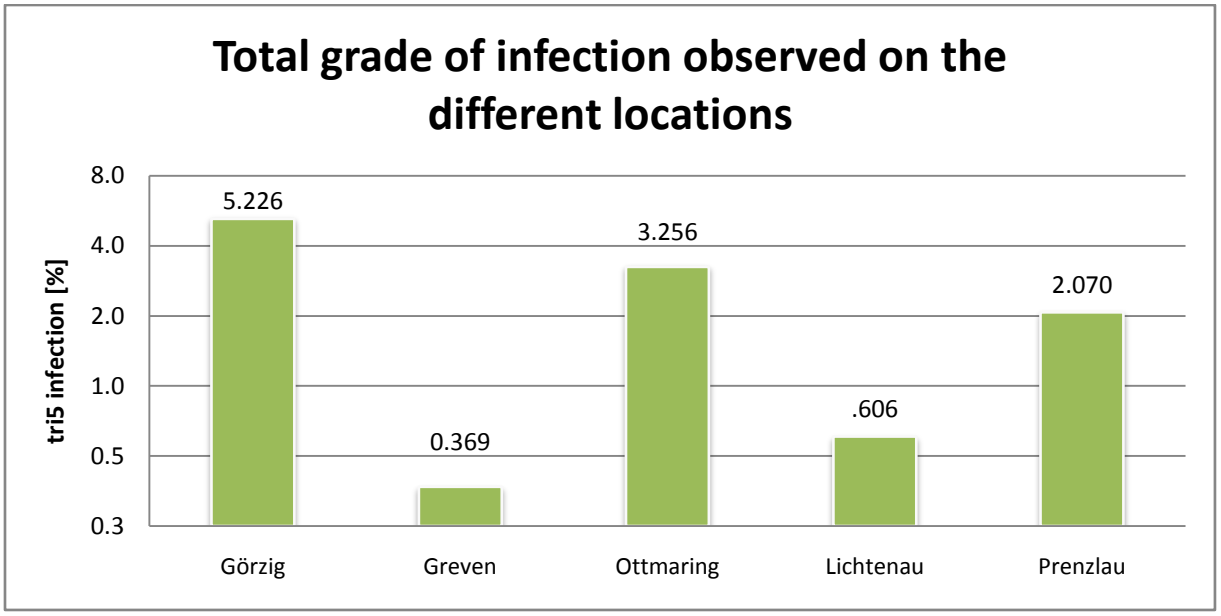


Figure 14: Comparison of the infection with trichothecene producing strains on the locations included in this study.

4.3.2. The *Fusarium* infection of the different varieties on different locations

To get a deeper insight in *Fusarium* susceptibility or resistance of the tested varieties, the mean values for the three field replications were calculated (see figure 15). The infection grade for each variety varies highly. The varieties Zidane (Zi), NK Nekta (NK) and LG3220 show in every location a general higher infection grade as other varieties. PR39F58 (PR) shows in all locations except Görzig a lower infection grade as other tested varieties. It must be noted that Ricardinio (RI) is very low infected in Prenzlau (0.12%) but shows moderate to high susceptibility on other locations. The mean values over the infection levels of each variety in all locations are illustrated in figure 16. These values give a good idea of resistance of a genotype, independent of the climate influence.

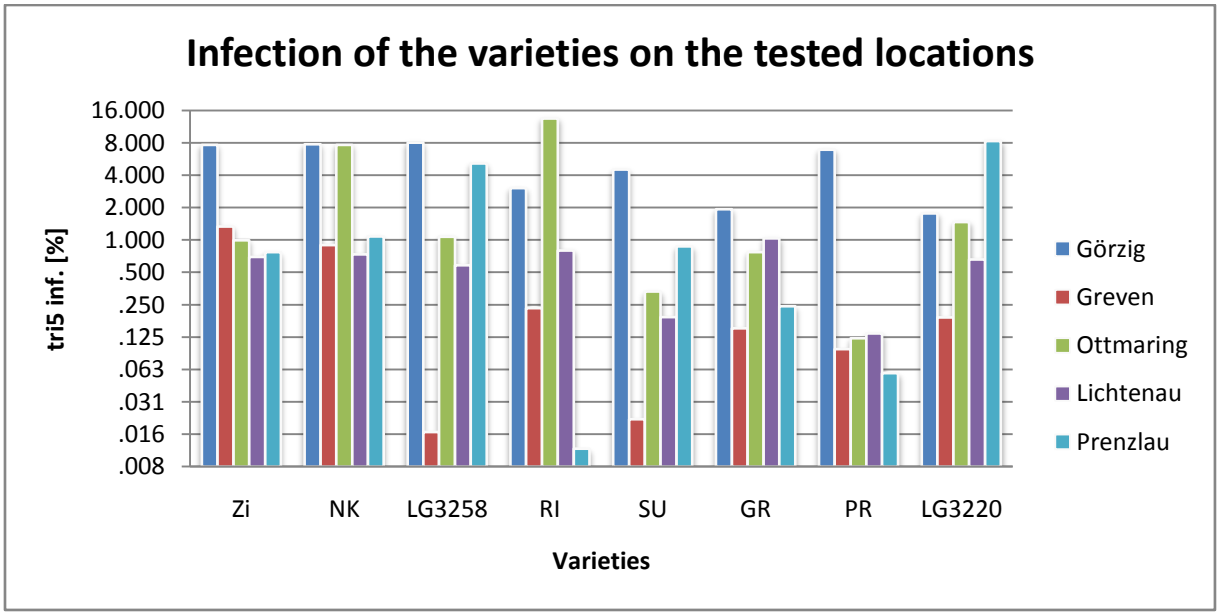


Figure 15: The bars show the *Fusarium* infection of the genotypes on all tested locations

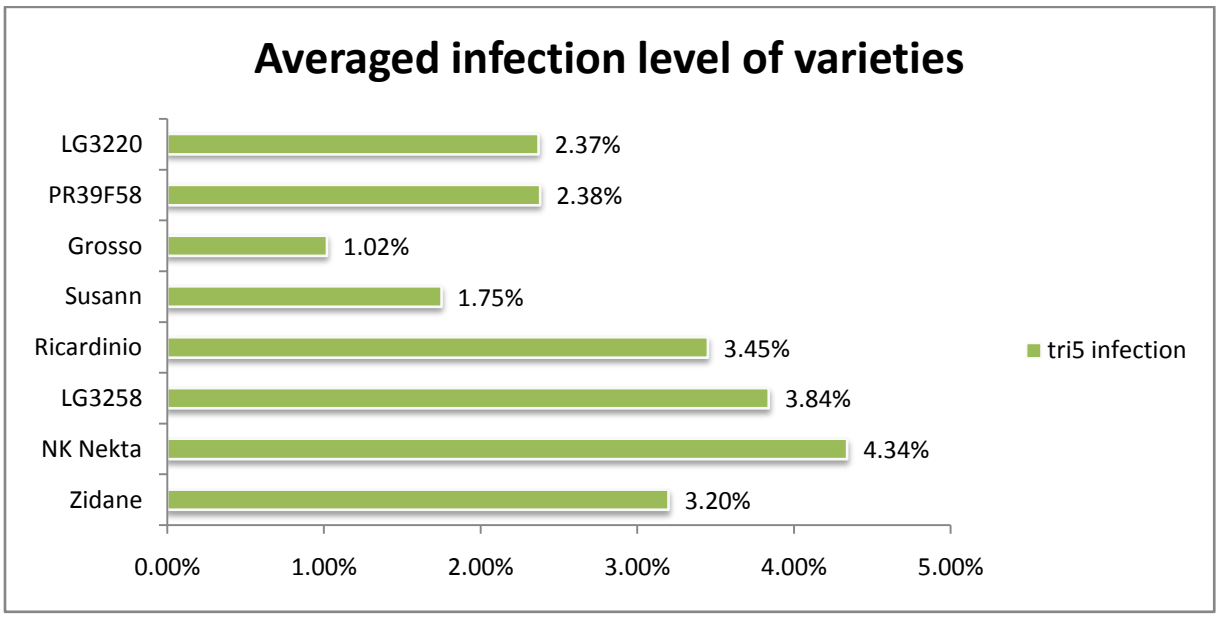


Figure 16: Averaged infection of varieties with trichothecene producing *Fusarium* strains

Grosso is with 1.02% infection the least infected variety in all locations. Strong infected are in contrast NK Nekta (4.34%) and LG3258 (3.84%). Although the samples were not artificially infected, all infection values are relatively high.

4.3.3. The analysis of field replications as example for the sampling problem

Five maize cobs were sampled for the repeated sampling whereas two charges consisting each of five maize cobs represent a field replication. The average values of the first and second sample hence represent the field replications. Every variety is planted three times on each location.

The infection grades of each field replication are illustrated in figure 17 for all locations. For facility of inspection, the infection grades are combined for each location in the graph 17. Particularly well match the three field replications of the varieties Susann (Su) and Grosso (Gr).

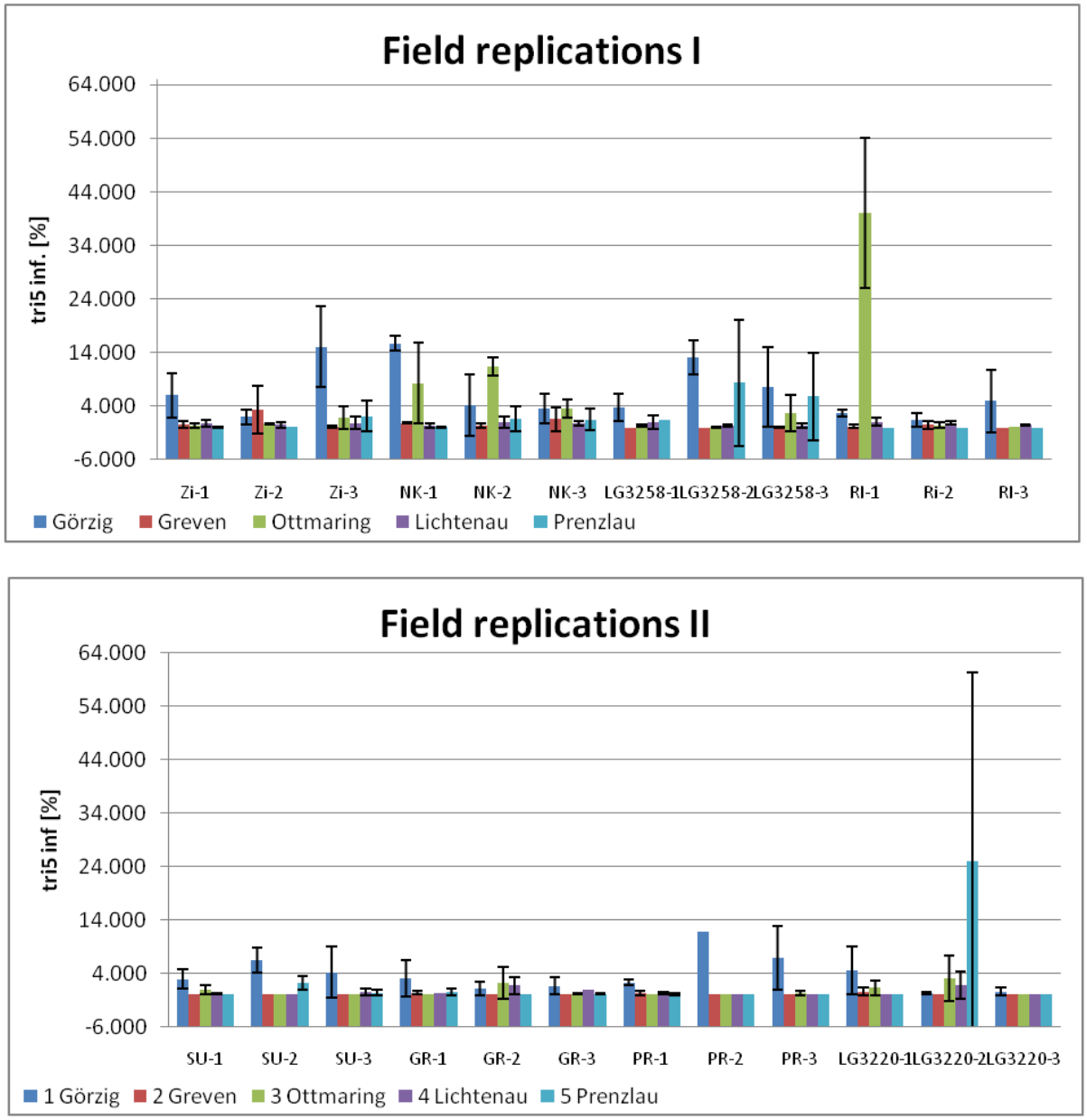


Figure 17: Field replications. The infection grade is shown for each field replication.

The standard deviations are very high as it is shown for the examples NK-2 (5.7%), NK-3 (2.7%) and LG3258-3 (7.4%). PR-1 (0.55%) and LG3220-3 (0.72%) show lower standard deviations.

4.3.4. Influence of the *adh1* incorporation on repeatability of the infection calculation with the reference gene system

In order to facilitate the comparison of results obtained from different DNA extractions a reference gene system was introduced by Brunner et al. [91] using as basis the method for genetically modified maize

analysis of the European Community Reference Laboratories (CRL) [95]. This protocol relates the fungal DNA to the total extracted DNA from a sample so that the reference gene system can compensate for differences in the DNA extraction efficiency. The gene for an alcohol dehydrogenase in maize (*adh1*) was used to determine maize DNA and the *tri5* gene was used as reference gene for trichothecene producing *Fusarium* strains.

It is necessary to evaluate whether the inclusion of the *adh1* measurement increases the error of the determination of *Fusarium* infection.
The *tri5* infection in percent was calculated by a formula introduced by Brunner et. al [91]:

$$Fusarium\ infection\ [\%] = \frac{tri5\ [ng]}{fum1\ [ng] + \ tri5\ [ng] + adh1\ [ng]} \times 100$$

Besides a relation of the *Fusarium* DNA to the total extracted DNA another method was frequently used in literature: the determined amount of *Fusarium* DNA can also be related to the amount of sample used for DNA extraction.

$$Fusarium\ DNA\ amount\ \left[\frac{\mu g\ DNA}{kg\ sample}\right] = \frac{tri5\ \left[\frac{ng}{\mu l}\right] x\ dilution\ factor\ x\ 100\mu l}{500\mu l}\ x\ 6000\mu l$$

The amount of *tri5* DNA was calculated in this formula. 6000µl is here for the volume of extraction buffer used for 1g of sample material. 500µl were transferred after the first centrifugation step and 100µl is the volume of finally added buffer.

The *adh1* values for Lichtenau are shown in figure 18. The *adh1* content for all samples from Lichtenau is very constant at a value of approximately 260 mg/kg (see figure 18).

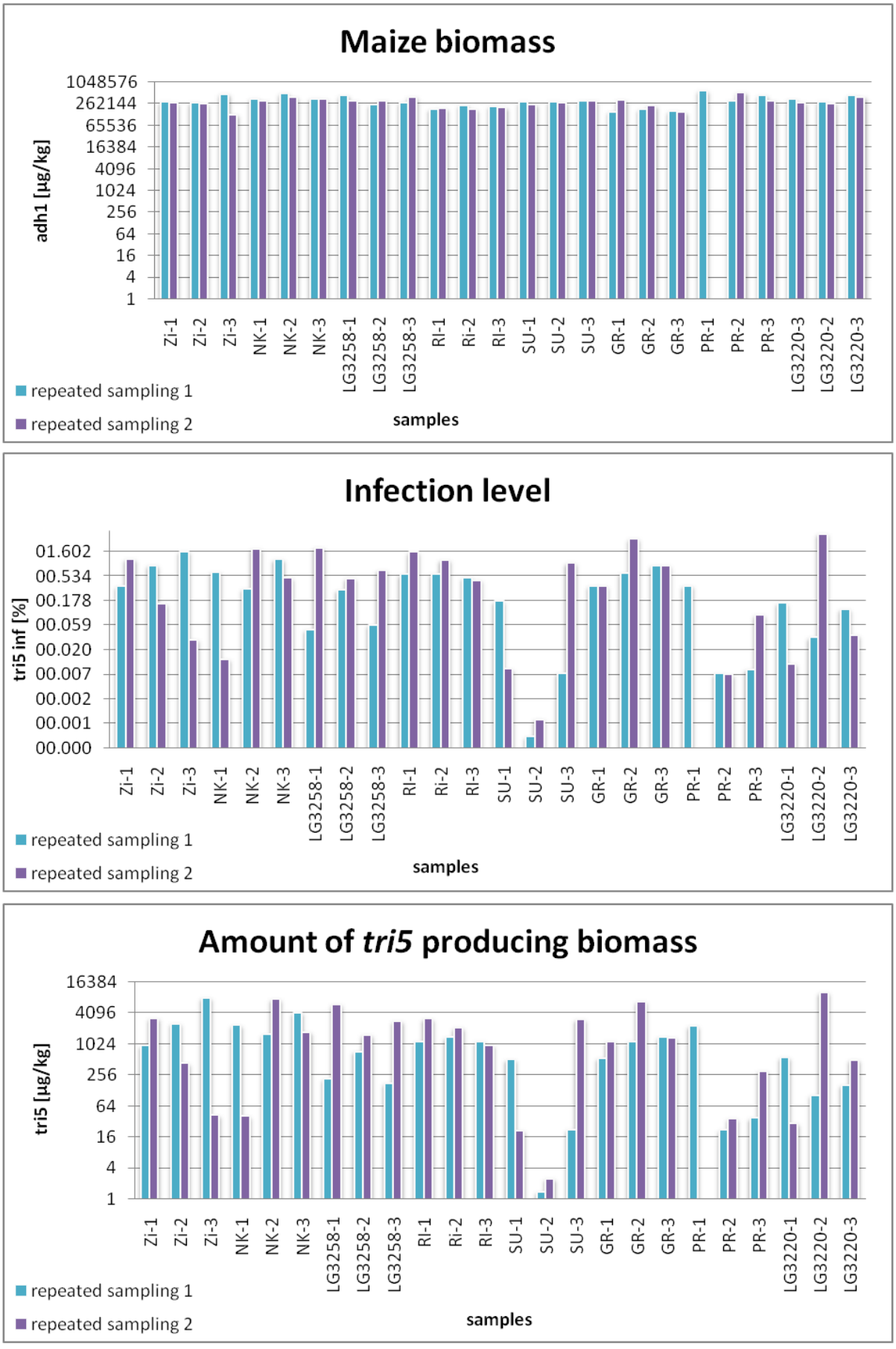


Figure 18: Comparison between maize content, infection levels and total *tri5* content in field samples of Lichtenau.

The measured infection in percent and the total amount of *tri5* producing biomass in µg/kg are shown in figure 18. As the samples located in Lichtenau show highly repeatable *adh1* values, also the values for the *tri5* infection in percent and the content of trichothecene producing isolates in µg/kg show similar patterns (see figure 18). The infection values are in a range of $4*10^{-4}$% to 3.6% and 1.37µg/kg to 10.31µg/kg, respectively.

The *adh1* values for samples from Ottmaring vary highly between two repetitions for a sample (figure 19). The *adh1* values reach from 1,286.9µg/kg to 993,870.5µg/kg. As the *adh1* amount of samples in Ottmaring varies highly, also the infection level and the amount of trichothecene producing biomass differ. An example for different trends between the infection in % and the *tri5* producing biomass in µg/kg is RI-1 (see figure 19).The first repetition has an infection grade of 30% and a *tri5* producing biomass of 26µg/kg; the second analysis of the same field replicate has an infection grade of 50% but only 1104µg/kg of *Fusarium* DNA. This difference can also be observed in the first field replication of the variety NK. The first sample is lower infected when the result is given in % (2.95% for the first sample versus 13.6% for the second sample) but has a higher *Fusarium* DNA content when the sample is shown in µg/kg (2,986µg/kg for the first sample versus 201µg/kg for the second sample). When the results are indicated as infection percent than the *adh1* value can adjust the *tri5* value as it is demonstrated for the sample Zi-2 in Ottmaring (see figure 19).

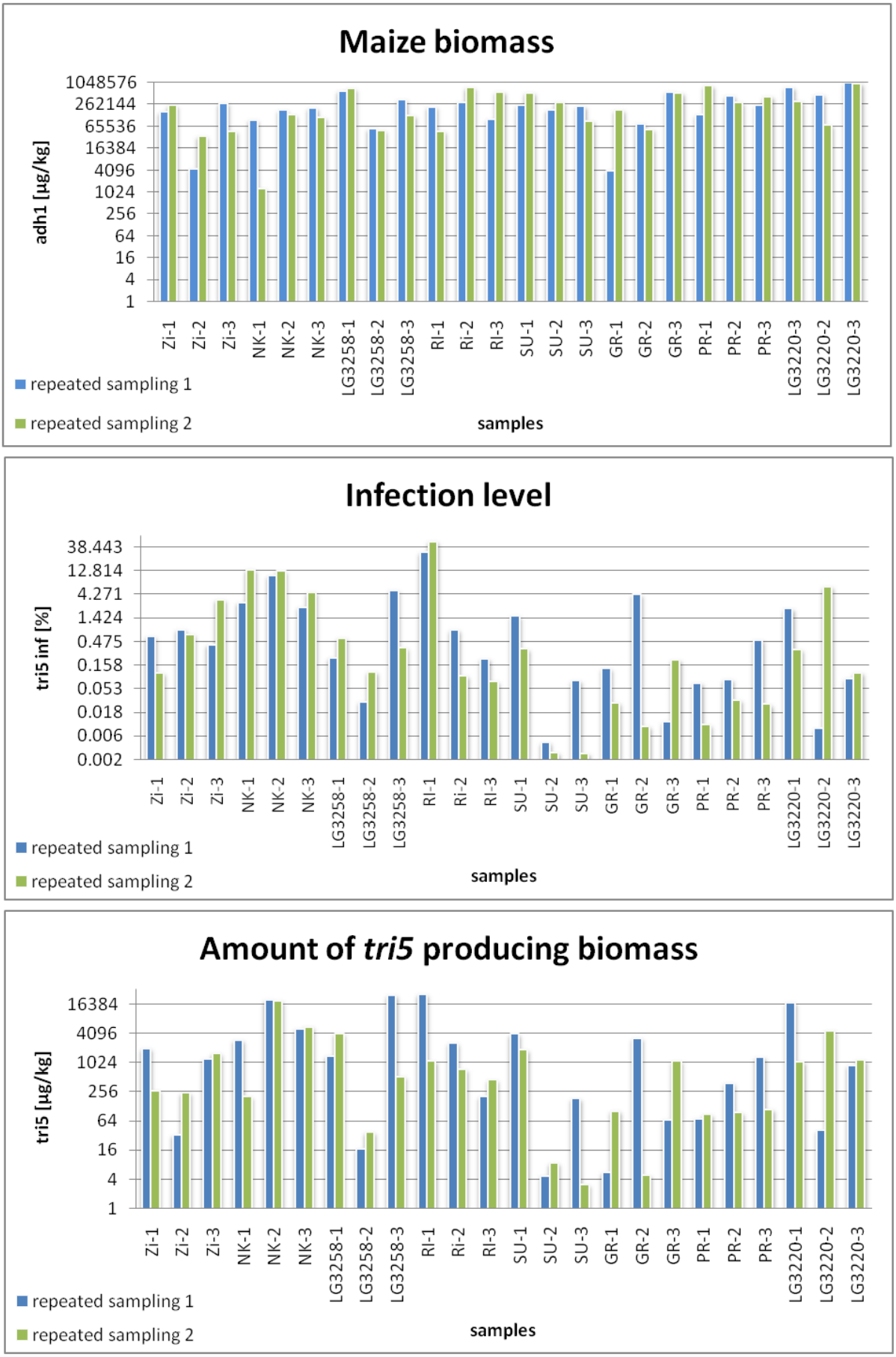


Figure 19: Comparison between maize content, infection levels and total *tri5* content in field samples of Ottmaring.

The *adh1* value for the first sample of Zi-2 (4,410.2µg/kg) is significantly lower than the second value (35,338.2µg/kg). To test whether the involvement of the *adh1* value would adjust the infection values, the *tri5* biomass in µg/kg is divided by the corresponding *adh1* biomass value. This means for the sample Zi-2 the following setting:

$$tri5\ \text{infection}\ of\ the\ first\ sampling\ [\%] = \frac{33.19\ \mu g/kg}{4410.2\ \mu g/kg} = 0.0075\%$$

$$tri5\ \text{infection}\ of\ the\ second\ sampling\ [\%] = \frac{242.17\ \mu g/kg}{35338\ \mu g/kg} = 0.0068\%$$

The difference between the fungal biomass of both repetitions (33.19µg/kg and 242.17µg/kg) is much higher than the difference between the calculated infection of 0.0075% and 0.0068%. The infection in percent differs only 9.3% whereas the difference between 33.19µg/kg and 242.17µg/kg is 86.29%.

Table 16: Mean coefficient of variation compared with infection level and *tri5* amount

	Mean coefficient of variation
Infection [%]	0.9
Total *tri5* amount [µg/kg]	0.05

4.4. Development of a quantitative triplex real-time PCR assay to measure trichothecene and fumonisin producing strains

The triplex assay is eminently suited to detect three fungal DNAs simultaneously using three different fluorescent dyes for the TaqMan® probes. The TaqMan® probe to detect the *tri5* gene of trichothecene producers was selected to carry FAM; the probe for the detection of the *fum1* gene from fumonisin producers carries the Cy5 fluorophor and the probe for the detection of maize DNA is labeled with JOE (see table 17).

Table 17 Fluorescent dyes with their maximum of absorbance.

DNA	**Fluorescent dye**	**Maximum of absorbance**
F. graminearum	FAM	495nm
F. proliferatum	Cy5	520nm
Maize	Joe	643nm

4.4.1. The development of the *fum1*/*tri5* duplex assay

The first step in the development of the triplex assay was to test whether a duplex assay for the detection of *fum1* and *tri5* would work. The primers were tested first for interactions by a real-time PCR assay with followed melting curve analysis. Then, different primer annealing temperatures and primer concentrations were tested (see table 18):

Table 18: Test of primer/probe concentrations and annealing temperatures. The concentrations are given in pM and the annealing temperature T_a in °C.

***Tri5* fw**	***Tri5* rev**	***Tri5* probe**	***Fum1* fw**	***Fum1* rev**	***Fum1* probe**	**T_a**	**C_T value**
100	100	50	100	100	50	56	27.34
						58	27.55
						60	27.16
						62	27.22
100	100	50	100	50	100	56	27.44
						58	27.19
						60	27.20
						62	27.16
100	100	50	50	100	100	56	27.82
						58	28.17
						60	28.32
						62	28.36
100	100	50	100	100	100	56	28.23
						58	27.14
						60	27.80
						62	28.15

It has been demonstrated that the primer concentration of 50pM *tri5* probe and all other oligonucleotides at 100pM gave the best results, the lowest C_T value, respectively. The optimal primer annealing temperature was 58°C.

Maize background DNA was spiked with 5ng/µl *F. proliferatum* DNA and 5ng/µl *of F. graminearum* DNA. This standard mixture was then measured with the Applied Biosystems cycler at an annealing and elongation temperature of 58°C (see table 19). All three DNAs were in the standard mixture,only the primer pair for the quantification of maize DNA was absent.

Table 19: Standard mixture of maize background with 5ng/µl *F. graminearum* DNA and 5ng/µl *F. graminearum* DNA.

Concentration of Standard DNA	C_T value for trichothecene producing isolates	C_T value for fumonisin producing isolates
50ng/µl	24.44	22.21
5ng/µl	27.80	25.68
0.5ng/µl	31.53	29.21

4.4.2. The developed of the *fum1*/*adh1* duplex assay

To develop a duplex assay for maize DNA and the DNA of fumonisin producers, both primer pairs were tested for interactions. An assay was therefore performed with *adh1* primers/probe and *fum1* primers/probe without DNA template but with a PCR mix containing SYBR Green. Subsequent melting curve analysis demonstrated that no dimers were formed between any of the components (see figures 20-21).

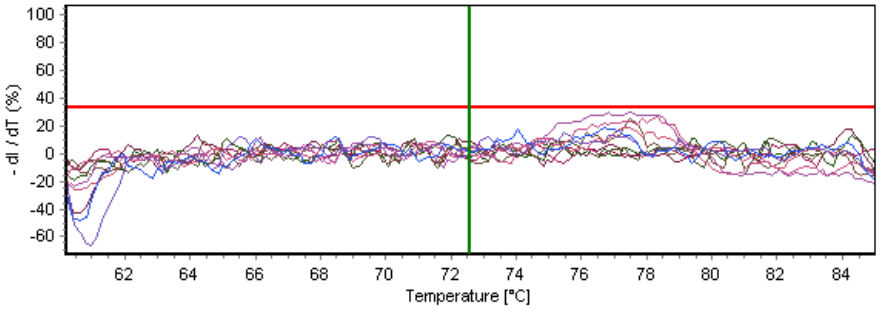


Figure 20: Melting curve analysis of a no template control run with *adh1* and *fum1* primers

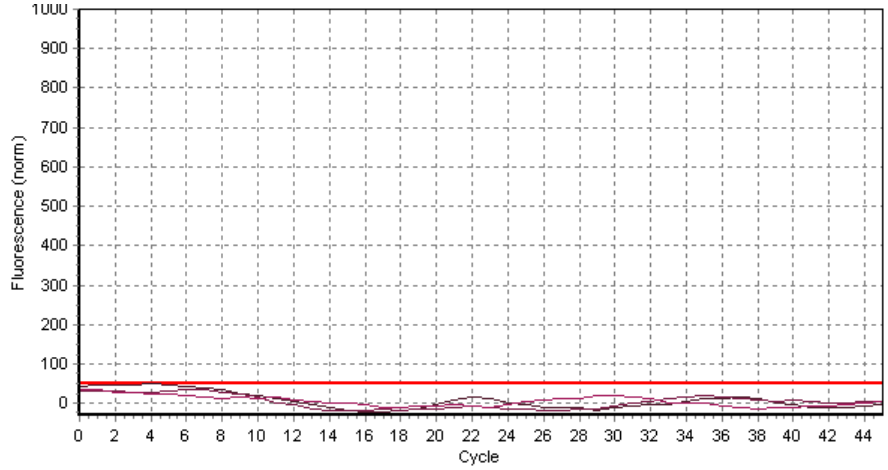


Figure 21: Assay with *adh1* and *fum1* primers; Kapa mix without SYBR was used to avoid unspecific detections caused by SYBR.

The duplex assay with maize and *Fusarium* DNA is shown in figure 22. A mixture of 45ng/µl maize DNA and 5ng/µl *F. proliferatum* DNA was measured. The amount of maize DNA was constant and the amount of *F. proliferatum* DNA was diluted tenfold by adding 50ng/µl maize DNA. The *adh1/fum1* assay was performed at 58°C (see table 20). It was known from the *adh1/tri5* duplex assay that the perfect primer concentrations are 100pM for *tri5* forward and *tri5* reverse primers and 50pM for *tri5* probe, *adh1* forward, *adh1* reverse and *adh1* probe.

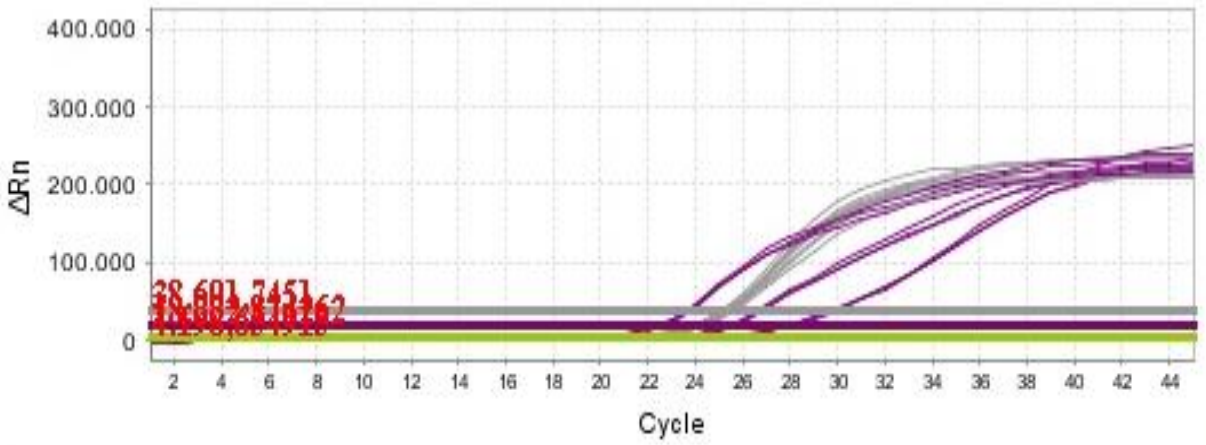


Figure 22: Duplex assay with maize DNA and *F. proliferatum* DNA.

Table 20: Standard mixture of maize background with 5ng/µl *F. proliferatum* DNA.

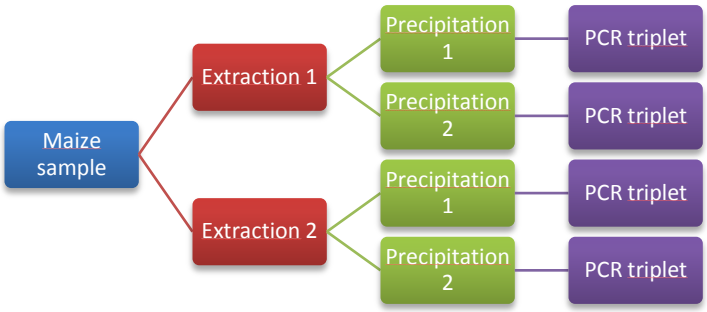
Concentration of Standard DNA	C_T value for constant amount of maize DNA	C_T value for fumonisin producing isolates
50ng/µl	24.54	22.11
5ng/µl	24.17	25.47
0.5ng/µl	24.25	29.23

4.4.3. The triplex assay

As all three basic duplex assays, namely the *tri5/fum1* assay, the *tri5/adh1* assay, and the *fum1/adh1* assay performed well, it could be expected that even the triplex assay will work properly. Three target DNAs (from *F. graminearum*, *F. proliferatum*, and maize) were mixed and detected at the same time with three different fluorescent dyes. A standard mixture was prepared to quantify the samples. 40ng/µl maize DNA was therefore mixed with 5ng/µl *F. proliferatum* DNA (*fum1* producer) and 5ng/µl *F. graminearum* DNA (*tri5* producer). The standard solution was tenfold diluted with maize DNA. The extracted maize samples were measured with a total DNA concentration of 50ng/µl.

4.4.4. Evaluation of the triplex assay on field samples

To analyze field samples with the developed triplex assay, the maize samples were extracted with the method A (CTAB extraction protocol). Two extractions were made for each sample on different days.



Schema 2: Analysis procedure for artificially infected maize samples.

Each sample was extracted twice with two DNA precipitations for each sample. This analysis procedure gives a detailed insight into repeatability of PCR analysis, DNA precipitation and the sampling which is applied for one extraction.

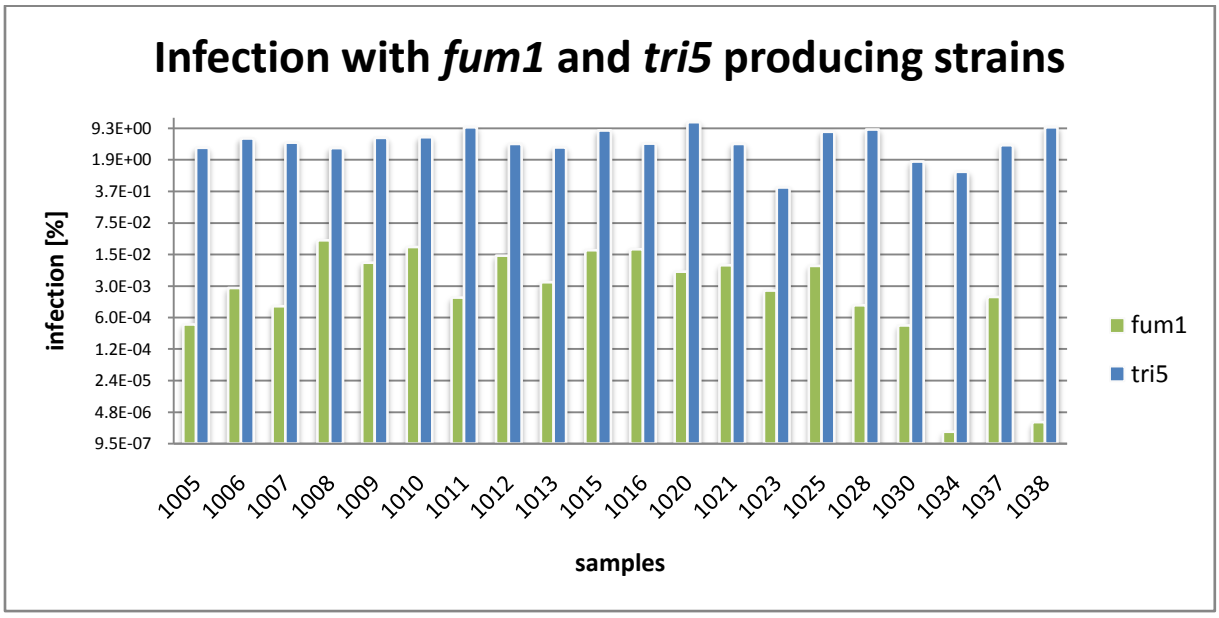


Figure 23: Infection of 20 maize samples with fumonisin and trichothecene producing *Fusarium* strains.

First of all, a mean value of all analysis was taken into account to observe a general abundance of the two toxin producers. The analyzed samples show a wide range of *Fusarium* infection, for both fumonisin and trichothecen producers (see figure 23). Generally, the infection with *fum1* producing *Fusarium* strains is between 1.8* 10^{-6} % and 3*10^{-2} %, whereas the range of the infection with *tri5* producing strains is much higher as the *tri5* infection values range from 0.46% to 12.64%.

The sample 1008 shows with 0.03% the highest infection with *fum1* producing strains whereas the sample 1020 with 12.64% has the highest infection with *tri5* producing strains. The lowest infection has the sample 1023 and shows only 0.46% proportion of *tri5* producing strains and 1034 (0.0000018%) for the *fum1* producing strains. The coefficient of variation for *F. proliferatum* infection is 0.67. The standard deviations vary from 1.7*10^{-6} % for the sample 1034 to 8*10^{-3} % for sample 1015. The standard deviations

for the *F. graminearum* infection reach from 1.9% for the sample 1006 to 0.1% as for the sample 1023. The variation coefficient is 0.16 for the *F. graminearum* infection.

To get an idea of the repeatability of each single step of the whole procedure the results of figure 23 are analyzed in further detail: mean values are broken apart into the separate extractions (figures 24-25) and then even into the two DNA precipitation steps (figure 26).

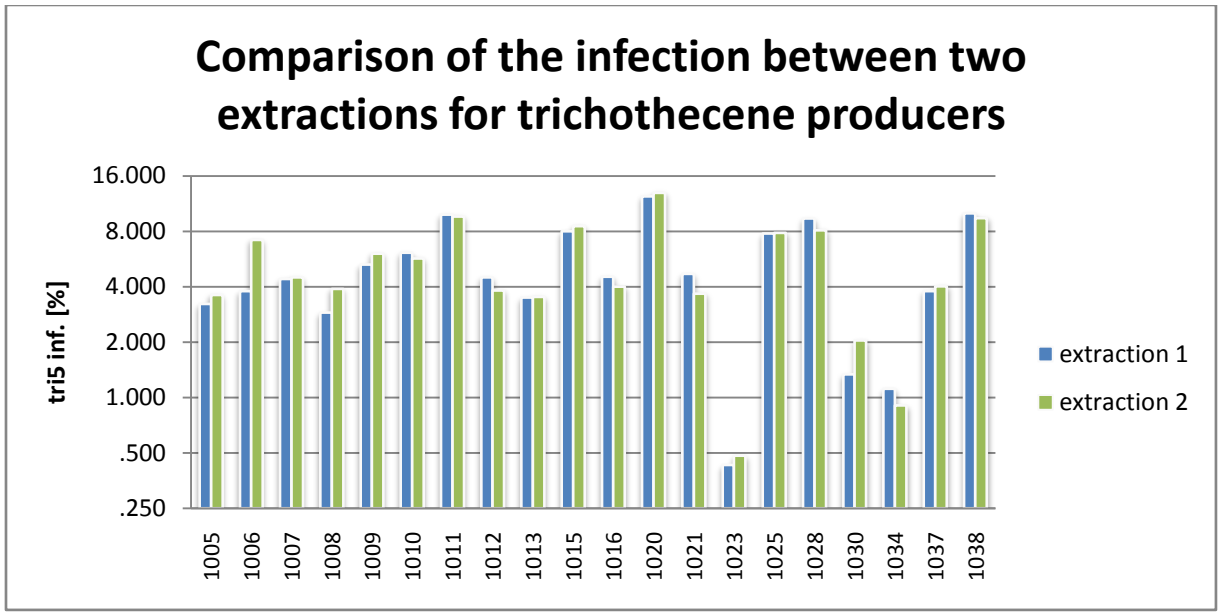


Figure 24 Comparison between the single extractions of each sample.

The sample 1005 in the *F. proliferatum* extractions is an appropriate example for a sampling problem (see figure 25); the infection grade of the first extraction is almost 24-times higher than that for the second extraction. The second extraction of sample 1006, which is considered to be the worst matching sample for the *F. graminearum* values, is just 1.9-times higher than the first extraction (see figure 24). The sample 1015 has the highest standard deviation with 2.05%; the sample 1023 has the lowest standard deviation of 0.1%.

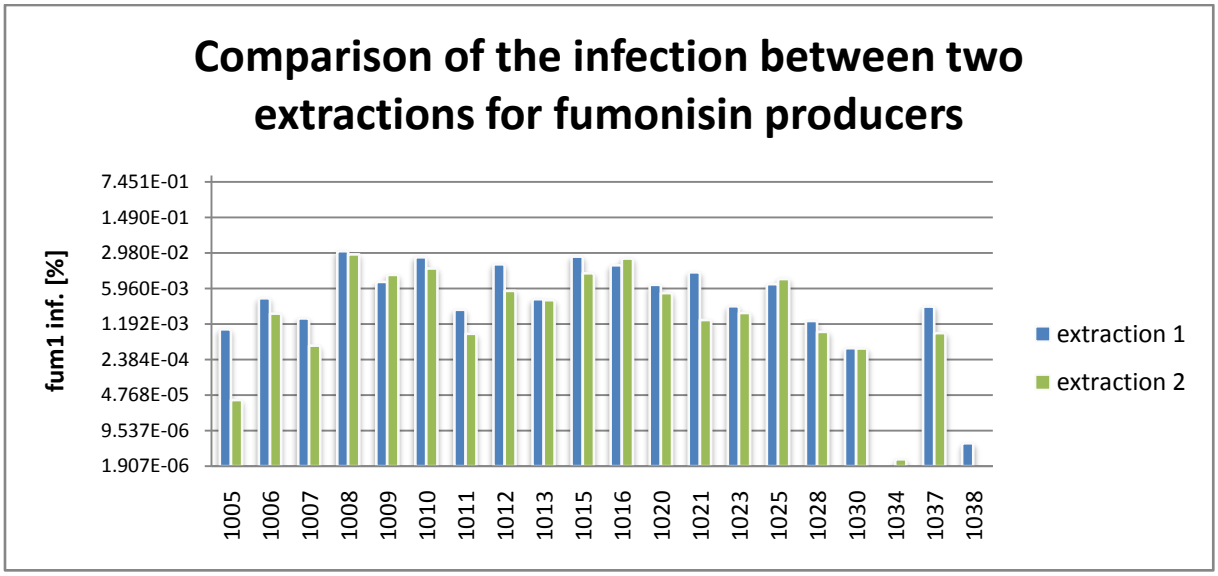


Figure 25 Comparison between the single extractions of each sample

In figure 26 are illustrated the precipitations to evaluate how well the single values match with each other.

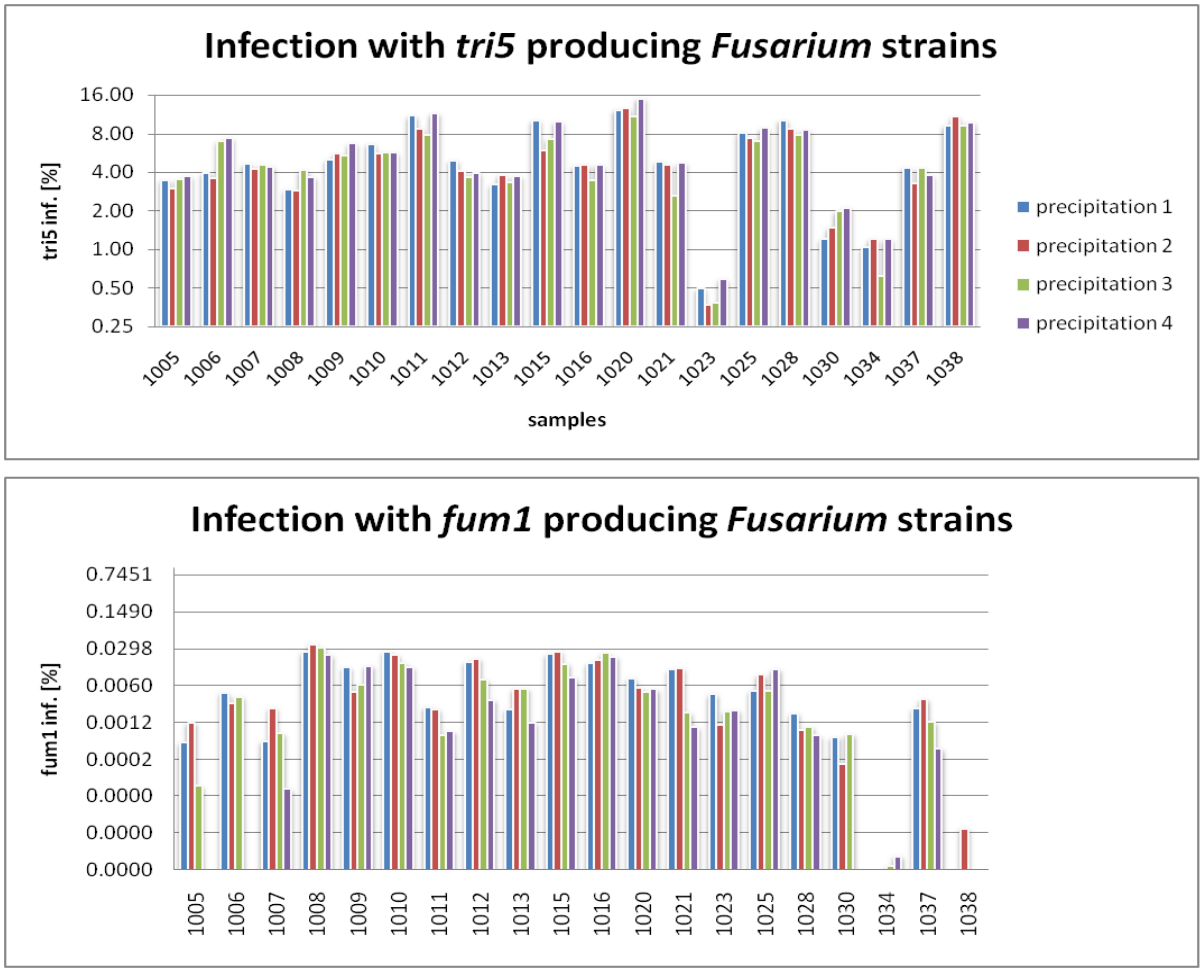


Figure 26: Comparison of the analysis of each single precipitation. In general it can be noted that the error caused by the DNA precipitation is only marginal.

A certain sampling problem is visible for the sample 1008 in *F. graminearum* infection (see figure 26). The two precipitations from the first extraction and the two precipitations from the second extraction match perfectly, whereas the two extractions vary.

4.4.5. Comparison of the PCR determined infection with the toxin content of the samples

The results of real-time PCR analysis were plotted against results gained from LC-MS/MS mycotoxin analysis (see figure 27-28). The correlation of the DNA content detected in the real-time PCR coincides with a stability index of 0.62 with the total amount of fumonisins if two values - designated as outliers - were eliminated. Without this elimination the correlation would be just 0.42.

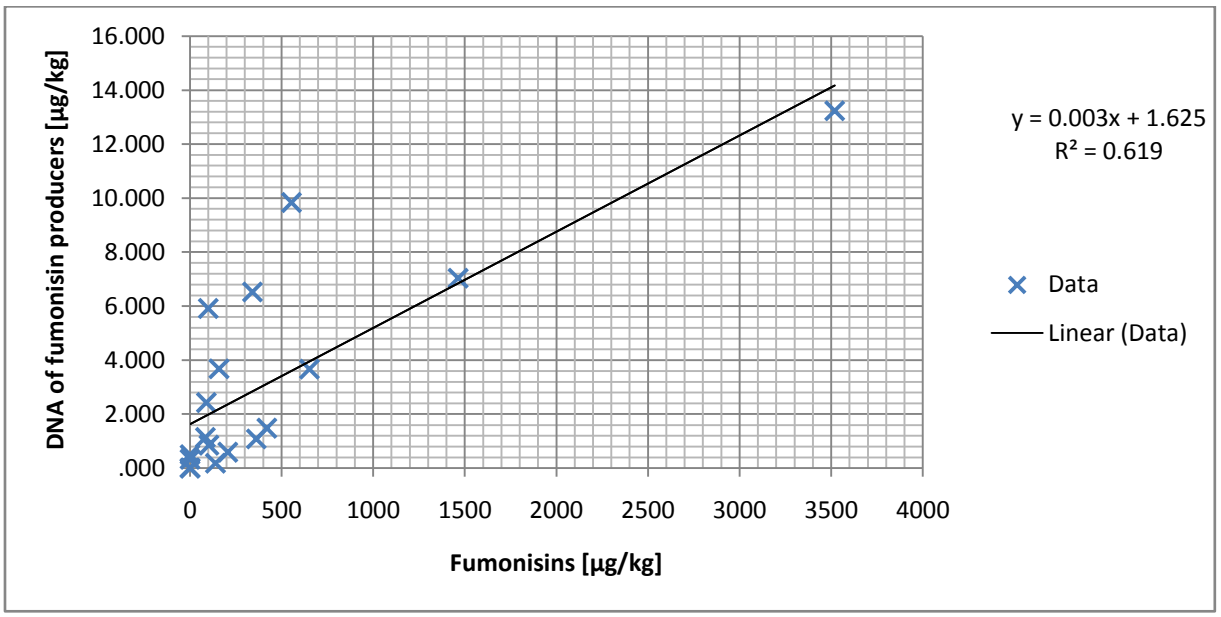


Figure 27: Total fumonisin content measured by LC-MS/MS analysis compared with *the DNA* content of fumonisin producing strains measured by real-time PCR. The amounts of total fumonisins include FB1, FB2, and FB3.

The correlation between the DNA content detected by real-time PCR and the total trichothecene content detected in LC-MS/MS is nevertheless quite well with a stability index of 0.77 (see figure 28).

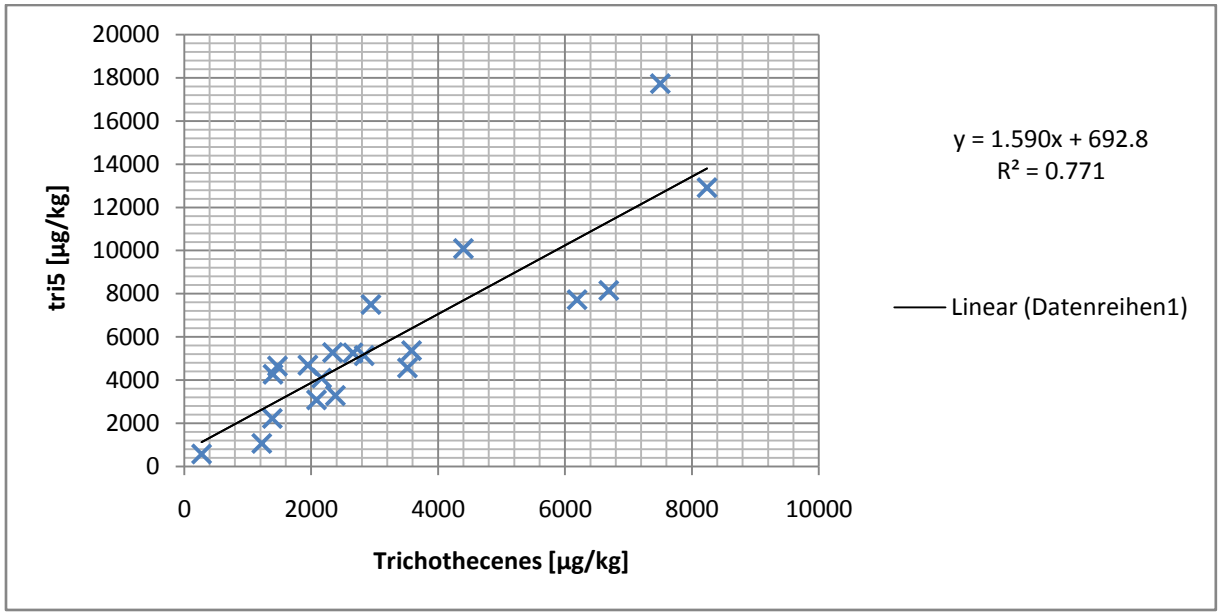


Figure 28: Total trichothecene content measured by LC-MS/MS analysis compared with the DNA content measured in real-time PCR.

4.4.6. Comparison between the results obtained by two different real-time PCR cyclers

The standard mixture for the triplex assay consisting of maize DNA, *F. graminearum* DNA and *F. proliferatum* DNA was analyzed with both Applied Biosystems 7500 and Rotorgene Q cycler. The coefficient of determination was 0.97 for trichothecene correlation and 0.99 for the fumonisin correlation (see figure 29).

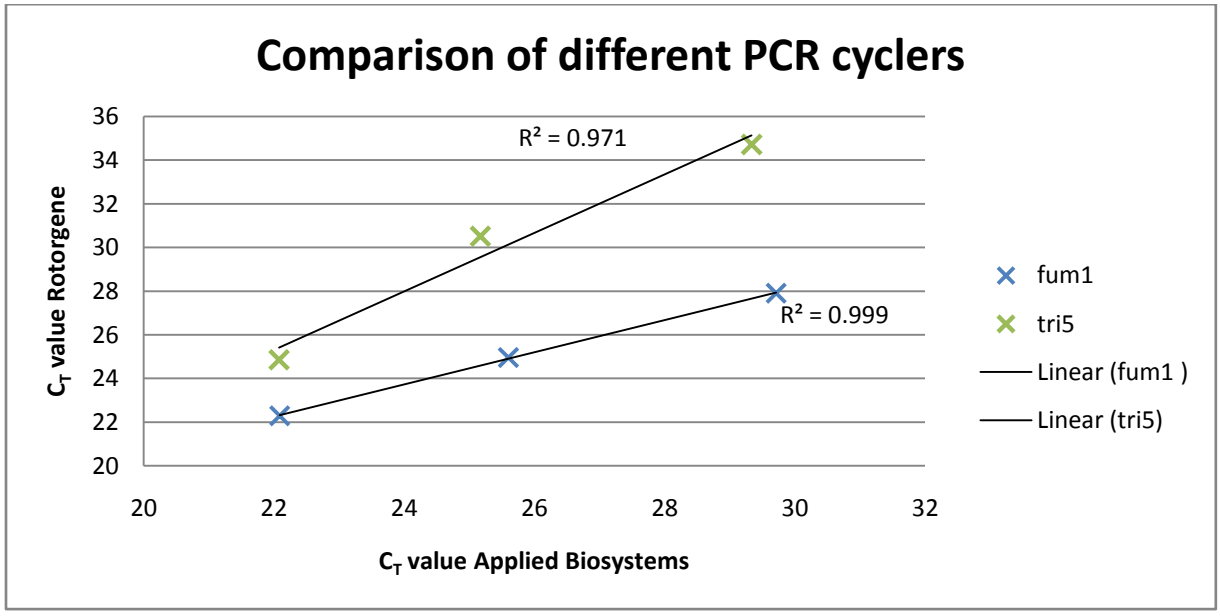


Figure 29: Comparison of the results obtained with the Applied Biosystems 7500 Fast Real-time PCR System and the Rotor-Gene® Q cycler.

5. Discussion

5.1. Isolation and purification of DNA for real-time PCR standards

In general, the standards used for quantification determine essentially the trueness of the obtained results for any analytical method. For numerous procedures, certified reference material is commercially available. However, DNA with controlled quality cannot be obtained for any real-time PCR target and especially not for niche applications like plant pathology. This means that the quality of the quantification is based on self-made DNA standards as the reference material is simply isolated from an organism by a suitable protocol, purified, quantified photometrically and then subjected to real-time PCR.

The main problem that might occur is fragmentation of the isolated DNA. This fragmentation results from a combination of enzymatical degradation during the isolation and a mechanical degradation due to pipetting steps or freezing and thawing. The factors causing the fragmentation cannot easily be controlled; only purification procedure can be applied to remove short DNA oligonucleotides and even single nucleotides from the longer fragments. If the average fragment length of standard DNA is close to or below the length of the diagnostic sequence (amplicon) a remarkable portion of the applied DNA is not PCR active anymore. The fragments are too short as both primers can bind on a fragment. To control this effect the DNAs were loaded onto an agarose gel to observe the grade of fragmentation.
F. nygamai, F. proliferatum 23 and *F. proliferatum 353* gave surprisingly high C_T values in the real-time PCR because of the high grade of fragmentation. *F. verticillioides* and *F. proliferatum* 2763 which gave the desired low C_T values in the real-time PCR were almost not fragmented as it can be seen on the gel (see figure 12).

The fungal and maize DNAs were purified with ultracentrifugation tubes (100kDa and 300kDa cutoff). Nevertheless, a cutoff over 10,000bp for the 300kDa devices was too high as fragments of this length are definitely PCR active and so most of the isolated DNA would get lost. The best results were obtained with 100kDa devices when a single wash step was performed after the normal protocol for the centrifugation devices. The loss of DNA amount was too high when the samples were washed twice with TE buffer. After purification with the centrifugation devices, almost no fragmentations could be observed anymore.

5.2. The detection of *tri5 producing Fusarium* DNA in a maize background

Naturally infected maize samples were planted in five locations distributed all over Germany. The *tri5/adh1* duplex was used for the detection of the infection levels.
The analysis of all samples revealed that the infection on the different locations varied significantly. Görzig shows the highest infection level of a mean of 5.23% over all planted maize genotypes whereas Greven has the lowest infection level with a mean of 0.37%.

It is interesting that the highest infected location and the least infected location are in West Germany and East Germany respectively. A closer look to the weather conditions of the sampling year 2010 shows that it was wet especially in Saxony-Anhalt from July to September (information about the climate conditions in 2010 was obtained from the German meteorological service: www.dwd.de). The maize samples located in Görzig got therefore more rain compared to other years or other places in Germany like Baden-Württemberg where the August was drier as in the rest of Germany. The dry conditions of August could result in the lower *Fusarium* infection levels of samples from Lichtenau.
An environmental indicator for the *Fusarium* infection could also be the storm force as conidiospores are distributed and settled on soil or cobs.

Eight varieties were planted under field conditions in five locations all over Germany. The real-time PCR analysis brought forward that none of the varieties is the best or the worse generally if every location is taken into account. Görzig has averaged the highest infected varieties with nearly 2% *F. graminearum* infection for the least infected variety (LG3220). The highest infected variety in Görzig is LG3258. LG3258 is in contrast the least infected variety in Greven whereas Zidane has the highest infection grade. PR39F58 is the most infected and Ricardinio is the least infected variety on this location. PR39F58 is least infected in Lichtenau whereas Grosso is high infected. It is to say, that Lichtenau has the most balanced distribution. The infection grades reach from 0.13% for PR39F58 to 1% for Grosso. LG3220 (8%) is the highest infected variety in Prenzlau whereas Ricardinio (0.12%) is the least infected variety in Prenzlau.

It could be assumed that the infection level might correlate with the starch amount of the variety but no evidence could be found for this assumption as highly starch producing maize varieties as Susann (1.75%), LG3220 (2.37%) and PR39F58 (2.38%) did not show an averaged higher infection level as other maize varieties. NK-Nekta (4.34%) has a normal starch production and has in average the highest infection level of all varieties.

The harvesting time could also play an important role for the fungal infection level. However, used samples were harvested on the same day. Basically it is to say that all tested varieties are early harvesting crops. The small variations in the perfect harvesting time did not lead to any increase or decrease of the

infection level (information about the varieties is obtained by syngenta: http://www.nk.com and KWS Saat AG: www.kws.de, Saaten Union: www.saaten-union.com).

Differences between varieties could also base in the distinct ability in the drying process. Some varieties dry particularly fast. The storage of maize and other grains is generally important as under wet storage conditions the fungi can grow further.

It is lastly assumed that the environmental conditions, like precipitation and temperature on the location are more important for the infection grade then the resistance itself. However, resistance is a trait that can actively be influenced by breeding but the climate has to be considered as an unchangeable fact.

Fusarium infections are known to be unequally distributed over fields. To get a deeper insight into this topic the three field replications of each variety were analyzed more in detail.

Concerning the results of the PCR determined infections it is obvious that in some locations the infection values correlate better than in other locations. Interestingly, in the least infected location, Greven, the field replications correlate best. A reason for this could be that the soil properties on this location are homogeneous. The samples could be also more evenly distributed due to the negligible infection grades. The chance to extract maize kernels that contain a low amount of fungal biomass is higher in marginal infected samples than in high infected samples. Exactly that can be observed when comparing the results of Greven with the field replications in Görzig. Görzig is the highest infected location and also the results from the three field replications vary for each variety. This relatively strong variation could derive from a sampling problem.

Two times five maize cobs were sampled for each field replicate. The averaged values over the two samples give the values for the field replications. Long-term studies are recommended to avoid a false interpretation of the diversities between the parcels per location.

To normalize the results obtained from DNA extractions a reference gene system was introduced by Brunner et al. [91] referring the fungal DNA to the total extracted DNA from a sample. Accordingly, the reference gene system can compensate differences in the efficiency of the DNA extraction. The gene for alcohol dehydrogenase in maize (*adh1*) was used to determine the maize background DNA.

The consideration of *adh1* as an additional value might improve the results but also bears the risk of producing an additional error. The coefficient of variation for the reference gene method is over all measured samples 0.6 whereas the coefficient of variation is 0.7 when *adh1* is not included into analysis. It

is beneficial to use the percent infection grade as when the extraction protocol gets modified, the results can still be compared as the reference gene compensated for different DNA yields.

5.3. The development of a comprehensive triplex assay for *Fusarium* detection

The above applied duplex assay to determine trichothecene producing species together with a reference gene should be expanded to include the analysis of fumonisin producing species. This triplex assay allows the screening for all *Fusarium* species producing the most relevant mycotoxins. The fumonisin producers can be measured by the amplification of a key gene in the fumonisin biosynthesis pathway, namely *fum1*. As a few qualitative and even quantitative PCR assays for this gene have been published previously, these primers were tested first. It turned out that the primer pairs designed by Waalwijk [88] worked best.

A gradient PCR was performed with these primers where it was shown that primer worked best when the annealing temperature was set at 58°C. The elongation step was removed because it could be demonstrated that multiplex assays work best if a two step protocol is used. All five available *Fusarium* strains could equally be quantified with these *fum1* primers. Dilution series were made for each *Fusarium* strain and all calculated efficiencies were between 0.85 and 0.92. It was important to demonstrate that the selected primer pair works with a similar efficiency for each strain to avoid strain specific differences in quantification

To set-up an appropriate triplex assay it was first shown that it is possible to combine all primer pairs for the detection of fumonisin producing and for trichothecene producing *Fusarium* strains. The optimal annealing temperature and primer concentrations were determined to run the assay with maximum efficiency for all three primer pairs.

To test the developed method in practical experiments, artificially infected maize samples cultivated in Styria were later used. The assay performed well, in all samples both DNAs from trichothecene and fumonisin producers could be detected. The values for the infection with fumonisin producers show high standard deviations due to the very low amount of DNA detected in all the samples. The values for the infection with *fum1* producing strains were from 0.0000018%- 0.03% whereas the infection values for *tri5* producing strains were from 0.46%-12.64%. Due to the extremely low level of infection the repeatability of the *fum1* analysis was lower than for *tri5* measurements. The two precipitations of the first extraction and the two precipitations of the second extraction match well, but comparing the two extractions with each other makes obvious that there is a significant difference. Most probably this error is caused by sampling for the two different extractions. Low fungal biomass concentration is often accompanied with an indifferent distribution pattern. The error could be decreased by increasing the sample volume for the extraction or to extract each sample tenfold.

The real-time PCR results for both *F. proliferatum* and *F. graminearum* infection did correlate very well within the two cyclers Applied Biosystems 7500 and Rotorgene Q. The coefficient of determination for the *tri5* measurement was 0.97 for *tri5* assay and 0.99 for the *fum1* assay. The well matching correlation was expected as both cyclers are state of art and the results should not differ between the cyclers.

5.3.1. Comparison of the real-time PCR determined infection with the toxin content of the samples

The LC-MS/MS obtained amounts for the mycotoxin content were compared with the real-time PCR results. The LC-MS/MS results were obtained by an acetonitrile/water/acetic acid (79/20/1) extraction. 5µl of the extract were injected into the HPLC and measured by the MS-group of the Center for Analytical Chemistry at the IFA Tulln.

The correlation between the two methods can be considered as good as the obtained correlation factor of 0.77 is equal to the results obtained in previous studies The correlation of the *fum1* assay with the fumonisin content is lower. It arises from the fact that a strain which is present in a low amount could produce a high amount of mycotoxins whereas another strain which is present in a higher biomass concentration produces a lower amount of mycotoxins.

Appendix

Abstract

Plant pathogenic fungi like *Fusarium* ssp. cause high economic impacts worldwide. These fungi are found on grains like wheat, maize and barely where they cause severe diseases like *Fusarium* head blight (FHB) or *Fusarium* ear rot. No total resistance could be achieved hence, as the genes providing resistances to *Fusarium* are located in distinct genomic regions. Based on the economic impacts it is important to investigate on revised methods for the detection of produced mycotoxins and the fungal biomass respectively. An appropriate method for the detection of mycotoxins is LC-MS/MS whereas the fungal biomass can be detected by real-time PCR.

Initially, a singleplex assay for the detection of fumonisin producing *Fusarium* strains was developed. Various primer pairs were tested on five *Fusarium* strains for the detection of the *fum1* gene encoding for a polyketide synthase. The most efficient primer pair has been found to be the primer pair published by Waalwijk et. al. [88]. These primers provided remarkable efficiencies (0.85-0.92) for all tested strains.

Then, two distinct duplex methods were developed for fumonisin producing strains in a maize background (*fum1*/*adh1* duplex) and both, fumonisin producing and trichothecene producing *Fusarium* strains respectively (*fum1*/*tri5* duplex). The gene for alcohol dehydrogenase *adh1* was used as reference gene for the detection of maize DNA while *tri5* was used for the detection of trichothecene producing *Fusarium* strains. The triplex assay was achieved by application of all three primer pairs for the detection of *fum1, tri5* and *adh1*. The triplex assay was evaluated by a set of 20 artificially infected maize samples. The obtained amounts of fungal biomass were compared to mycotoxin contamination obtained by LC-MS/MS method.

Due to the simultaneous detection of all three genes in a single run a considerable time- and cost reduction for the analysis of maize samples could be achieved.

Zusammenfassung

Bei der Gattung *Fusarium* handelt es sich um pflanzenpathogene Pilze, deren Mykotoxine in Verdacht stehen Krankheiten vor allem bei Nutztier aber auch bei Menschen zu verursachen. Der Pilz selbst ruft unter anderem bei Weizen und Mais Krankheiten wie die Ährenfusariose bzw. Kolbenfäule hervor. Durch *Fusarium* Stämme kommt es zu beachtlichen Ernteausfällen weltweit was zu einem großen ökonomischen Schaden führt. Aufgrund dessen ist es wichtig immer neuere und verbesserte Methoden zur Eindämmung des Befalls aber auch zur Detektion der Mykotoxine bzw. der Pilz-Biomasse zu entwickeln. Mit Hilfe von chromatographischen Messverfahren wie LC-MS/MS lassen sich die Mykotoxingehalte sehr gut bestimmen. Möchte man den biologischen Aspekt miteinbeziehen und die Biomasse des pathogenen Pilzes bestimmen eignet sich dafür besonders die real-time PCR. In dieser Arbeit wurde mit dem Nachweis des Genes *fum1* begonnen. Dieses Gen ist essentiell für die Biosynthese der Fumonisine. Verschiedene Primer-Paare wurden hierfür getestet. Als das am besten geeignete stellten sich die Primer von Waalwijk et. al. [88] heraus. Sie lieferten sehr gute Effizienzen (0.85-0.92) für alle fünf getesteten *Fusarium* Stämme. Des Weiteren wurde die DNA von Standards zur Quantifizierung gereinigt um konstantere Resultate zu erzielen.

Eine früher entwickelte duplex Methode zum Nachweis von Trichothecen bildenden Pilzen wurde anhand eines großen Probensatzes evaluiert. Als Referenzgen für Mais dient das Gen für eine Alkohol-Dehydrogenase (*adh1*). Als Referenzgen für Trichothecen produzierende *Fusarium* Stämme wird *tri5* verwendet, welches für ein Enzym codiert, das den ersten Schritt im Biosyntheseweg der Trichothecene katalysiert. Indem zur duplex Methode ein weiteres Gen, *fum1*, hinzugefügt worden ist, kam es über die gemeinsame Detektion von *adh1* und *fum1* bzw. *fum1* und *tri5*, zur Entwicklung der triplex Methode. Die triplex Methode wurde durch einen Probensatz von 20 künstlich infizierten Maisproben evaluiert. Die erhaltenen Biomassewerte wurden mit Mykotoxinwerten aus der LC-MS/MS Analyse verglichen. Die Korrelation war bei *tri5* mit einem R^2 von 0.72 besser als bei *fum1* mit einem R^2 von 0.62.

In dieser Arbeit wurde eine real-time PCR Methode entwickelt um gleichzeitig drei unterschiedliche Gene zu detektieren. Durch die gemeinsame Detektion aller zwei Gene für die Mycotoxinbildung und eines Referenzgenes für Mais kommt es nicht nur zu einer Zeitersparnis sondern auch zu einer beachtlichen Kostenersparnis.

Danksagung

Zu Allererst möchte ich mich herzlichst bei meinem Betreuer am IFA Tulln, Dr. Kurt Brunner bedanken, der stets ein offenes Ohr für alle meine Fragen und immer eine kompetente Antwort parat hatte. Des Weiteren bedanken möchte ich mich bei meinen Betreuern auf der TU Wien, Prof. Robert Mach und auf der Universität Wien, Prof. Udo Bläsi. Bei Dr. Michael Sulyok möchte ich mich für die zur Verfügung gestellten Maisproben und die LC-MS/MS Daten bedanken. Ein Dank geht auch an die Abteilungen Pflanzenproduktion am IFA Tulln und das Institut für Verfahrenstechnik, Umwelttechnik und technische Biowissenschaften der TU Wien für die Pilzstämme.

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