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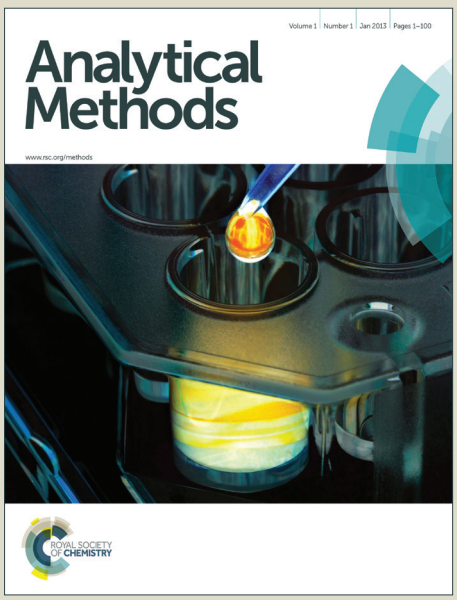

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Development of a multiplex real-time PCR to quantify *Fusarium* DNA of trichothecene and fumonisin producing strains in maize

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Contamination of cereals with *Fusarium* species is one of the major sources of mycotoxin contamination in food and feed. Despite great progresses in plant breeding, a complete resistance to *Fusarium* species has not yet been achieved. Visual scoring of disease symptoms combined with the determination of mycotoxins are common approaches to identify new *Fusarium* tolerant lines, but these methods are only indirect and therefore of limited use to determine the level of resistance against *Fusarium* spp.

Aiming at a rapid and sensitive quantification method for trichothecene and fumonisin producing *Fusarium* species in maize, a multiplex qPCR assay was developed. This method enables high-throughput screening of a huge number of samples for *Fusarium* infection in a relatively short time due to simultaneous quantification of the mycotoxin-related genes *tri5* and *fum1*. The multiplex method was applied to 24 maize field samples. All of these were analyzed for the trichothecenes deoxynivalenol (DON), DON-3-glucoside (D3G), nivalenol (NIV), 3-acetyl-DON (3-ADON), T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS) and neosolaniol (NEO) and the fumonisins fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) by LC-MS/MS and for the mycotoxin producers by the new qPCR multiplex assay. The assay was found to be specific for fumonisin as well as for trichothecene producing *Fusarium* species. The limit of quantification was found to be 0.32 pg per µl for both *Fusarium* strains. To our best knowledge this is the first report of the use of a multiplex qPCR for the quantification of trichothecene and fumonisin producing *Fusarium* species.

1 Introduction

One of the major problems in modern agriculture is the soil born plant pathogenic fungus *Fusarium* which causes considerable economic impacts worldwide.[1-4] The infection with this pathogen results in severe damages of numerous cultivable plants like maize, wheat and barley as the agronomical most important host plants.[5, 6] Due to the global infestation of crops with *Fusarium* species, significant yield and quality losses arise. The kernel size and weight is usually reduced upon infection with *Fusarium*. But even more important are the numerous toxic metabolites which are produced during the colonization of the plant. These compounds have been related to toxic effects upon ingestion by humans and animals.[7, 8] The most important trichothecene producing *Fusarium* species are *F. graminearum* and *F. culmorum*, whereas *F. verticillioides* and *F. proliferatum* are the major fumonisin producing species.[9-12] Mycotoxin levels in infected maize plants can vary significantly among maize cultivars and are usually higher in susceptible plants than in more resistant cultivars. Therefore, the determination of the resistance of new maize varieties is of high importance and usually combines the analysis of mycotoxins by enzyme linked immunosorbent assay (ELISA), high performance liquid chromatography (HPLC) coupled either to UV and/or mass spectrometric (MS) detection methods[13, 14] and the visual scoring of disease symptoms.[15] Methods to determine mycotoxin contents are highly sensitive but nevertheless time-consuming, cost-intensive (especially HPLC/MS) and only indirect, because they provide no information about the actual biomass of a fungus in a sample. Hill et al.[16] developed a *Fusarium*-specific ELISA to directly determine *Fusarium* biomass. The authors analyzed artificially infected barley samples and found out that fungal biomass determination requires only one third to one fourth of the field replicates to acquire the same information on plant resistance as with visual scoring or DON analysis. Another method to determine the fungal

biomass during infection of a plant deals with the measuring of the ergosterol content of cereal samples.[17-19] The analytical procedure to measure the ergosterol levels is as elaborate as toxin measurements and therefore no commercially applicable alternative to determine the *Fusarium* resistance of a plant. Recent studies focused on the development of more direct techniques to quantify *Fusarium* diseases. In general, two methods gained acceptance in the last decades: immunoassays and polymerase chain reaction (PCR). ELISA allows the direct measurement of *Fusarium* species biomass in infested plant tissue due to specific fungal antigens. It profits from its low costs and ease of sample preparation. Nevertheless, commercial antibodies for *Fusarium* are rare. Another interesting novel method to gain direct information about the biomass of a fungus is the quantitative PCR (qPCR) technique, which is based on the quantification of the amount of organism specific DNA.[20-22] An infection of highly resistant plants can so be detected before any symptoms are visible.[20, 23] Besides the application as a rapid and therefore inexpensive method for resistance evaluation, the qPCR can perfectly be applied for *Fusarium* monitoring projects and even as a screening method for food and feed contamination.[24] Furthermore, the PCR has the potential to analyze numerous samples in parallel, and common PCR instruments even allow the analysis of approximately 45 samples in duplicates within less than one hour. Additionally to existing species specific assays[20, 21] tests were developed to quantify all trichothecene producing *Fusarium* species[22] or all fumonisin producing *Fusarium* species[25, 26] in one assay by the detection of a gene essential for the respective mycotoxin production. However, to obtain reliable results an elaborate optimization of the whole procedure is indispensable.

Bluhm et al.[25] developed a multiplex PCR assay that provides differential detection of fumonisin and trichothecene producing *Fusarium* species. Nevertheless, this published method is only qualitative and allows no quantification of fungal biomass. In this study we describe for the first time the development and application of the quantification of three relevant target genes in parallel. The *tri5* gene, encoding for the fungal trichodiene synthase, was targeted to quantify DNA from trichothecene producing species. The trichodien synthase is the enzyme catalyzing the first step of trichothecene synthesis.[27] The *fum1* gene encodes for a polyketide synthase and has been applied to measure DNA from fumonisin producing *Fusarium* species.[25] According to Brunner et al.[28] a co-quantification of plant DNA compensates for variable DNA extraction efficiencies and is able to improve the repeatability of PCR tests. Therefore, the plant gene *adh1*, encoding for the alcohol dehydrogenase of maize was included in the measurements as a reference gene. In order to develop a quantitative multiplex group specific assay for the detection of trichothecene and fumonisin producing *Fusarium* species, primers and probes were used from previously presented studies[26, 28, 29] and new qPCR reaction conditions were developed to run all three assays in one reaction. The performance of the new method was evaluated with twenty-four maize samples collected in Austria and the novel triplex assay was compared to the three singleplex qPCR runs. Thereafter, all maize samples were analyzed for the trichothecene toxins DON, DON-3-glucoside (D3G), nivalenol (NIV), 3-acetyl-DON (3-ADON), T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS) and neosolaniol (NEO) and the fumonisins fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) by liquid chromatography tandem mass spectrometry (LC-MS/MS) and compared to the qPCR results.

2 Experimental

All chemicals and reagents used for extraction were obtained from Carl Roth GmbH (Karlsruhe, DE), whereas the primers and probes were ordered at Sigma Aldrich (St. Louis, MO). The Kapa qPCR master mix was obtained from PeqLab (Erlangen, DE).

2.1 Sample preparation

Twenty-four ground maize samples from four different locations (A, B, C and D) in Austria were obtained from the Center for Analytical Chemistry at the IFA Tulln. All samples were stored at -20 °C. The toxin contents of DON, D3G, NIV, 3-ADON, T-2, HT-2, DAS, NEO, FB_1, FB_2 and FB_3 were analyzed by LC-MS/MS and the fungal biomass was quantified by qPCR.

2.2 DNA extraction

The protocol used for the DNA extraction from maize kernels is a modification of a method recommended by the European Union Reference Laboratory (EURL).[30]
One g of ground maize sample was weighed into a 15 ml flask and 6 ml CTAB (cetyltrimethylammonium bromide) extraction buffer (1.4 M NaCl, 2 % w/v, CTAB, 0.1 M Tris-Base pH 8, 0.02 M EDTA pH 8, 1 % w/v polyvinyl pyrrolidone 40000), preheated up to 70 °C, were added. The samples were incubated for 1 h at 70 °C, mixing the tubes every 10 minutes and then the tubes were centrifuged at 2,978 x g for 15 minutes at room temperature (RT). Subsequently, 500 µl of the supernatant were transferred into a clean tube. Then 55 µl of 10 % preheated CTAB solution (10 % w/v CTAB, 0.7 M NaCl) and 550 µl of a mixture of chloroform:isoamyl alcohol (24:1) were added and the tubes were inverted 20 times. The samples were centrifuged at 7,200 x g for 5 minutes at RT. Thereafter 350 µl of the upper aqueous phase were collected and transferred into a clean tube and 1,050 µl precipitation buffer (1 % w/v CTAB, 0.05 M Tris-Base pH 8, 0.01 M EDTA pH 8) were added. The solution was mixed gently and was kept at RT for 30 minutes for precipitation. The DNA was then collected by centrifugation at 7,200 x g for 15 minutes at RT. The supernatant was discarded and the pellet was washed twice with 200 µl precooled 70 % ethanol. The pellet was then vacuum dried for 30 minutes at 37 °C and the DNA was re-suspended in 100 µl 10 mM Tris buffer (pH 8). Finally, the solution was incubated in a thermomixer for 30 minutes at 65 °C to ensure solubilisation of the DNA and stored at -20 °C.
Maize genomic DNA was used as DNA standard. It was isolated from freshly grown maize leaves as described by Saghai-Maroof et al.[31] DNA from *F. verticillioides* and *F. graminearum* used as fungal DNA standards were obtained by a phenol-chloroform extraction according to Peterbauer et al.[32]

2.3 Real-time PCR primers and dual-labelled probes

Three sets of specific primer pairs and the appropriate probes were used to amplify either maize DNA, DNA from fumonisin or from trichothecene producing *Fusarium* species. The *adh1*, *fum1* and *tri5* probes were labeled at the 5'-ends with JOE (6-Carboxy-4', 5'-Dichloro-2', 7'-Dimethoxyfluorescein), Cy5 (Indodicarbocyanine) and 6FAM (6-Carboxyfluorescein), respectively and on the 3'-end with a quencher (Black Hole Quencher® 1 – BHQ1). The primers adh1_fw (5'- CGTCGTTTCCCATCTCTTCCTCC-3') and adh1_rev (5'- CCACTCCGAGACCCTCAGTC-3') specifically amplified an 136 bp fragment of the *adh1* gene of maize, which was quantified by the dual labelled probe adh1_probe (JOE-5'- AATCAGGGCTCATTTTCTCGCTCCTCA-3'-BHQ1). The *Fusarium* DNA of trichothecene producing species was detected by the amplification of an 178 bp fragment of the *tri5* gene. For the amplification and quantification the primer pair tri5_fw (5'- GATTGAGCAGTACAACTTTGG-3') and tri5_rev (5'- ACCATCCAGTTCTCCATCTG-3') as well as the tri5_probe, a locked nucleic acid (LNA) oligonucleotide (bases in brackets are locked), (6FAM-5'- C[+C][+T][+T][+G]G[+G]CCA-3'-BHQ1) were used. The primers and probes used for the *adh1* and *tri5* assay were published by the EURL[29] and Brunner et al.[28], respectively. The primer pairs fum1_fw (5'- ATGCAAGAGGCGAGGCAA-3') and fum1_rev (5'- GGCTCTCAGAGCTTGGCAT-3') as well as the fum1_probe (CY5-5'- CAATGCCATCTTCTTGAAACCT-3'-BHQ1) to quantify fumonisin producing species DNA were slightly modified from Waalwijk et al.[26]. The amplified fragment has a length of 149 bp.

2.4 Real-time PCR optimization and assay evaluation

Initially, the three primer sets were tested in separate reactions based on the EURL,[29] Brunner et al.[28] and Waalwijk et al.[26]. The optimization of the multiplex qPCR was performed by empirically varying critical factors that affect multiplexing such as primer concentrations and annealing temperatures. All analyses were performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) as well as on a Rotor-Gene Q (Qiagen, Hilden, DE) to test the reproducibility of the assay. A total reaction mix volume of 15 µl containing 2 µl of template DNA, 7.5 µl Kapa probe fast master mix, 4.78 µl/3.34 µl water (singleplex and multiplex, respectively) and 0.1 pM/µl or 0.05 pM/µl of dual labelled probe, forward and reverse primer was used. The PCR cycling conditions included an initial denaturation step at 95 °C for 1 min and 50 s, followed by 45 cycles of denaturation at 95 °C for 15 s and primer annealing and amplicon extension at 58 °C for 45 s. PCR reactions were performed in triplicates on all samples.
The cross-reactivity of the primers used in the developed multiplex qPCR method was analyzed by testing the different primer pairs on different fungal species. Therefore, *fum1* primers were tested for amplification of *tri5*

producing *Fusarium* strains and vice versa. The specificity of the *tri5* oligonucleotides was already evaluated by Brunner et al.[28]. To prove equal efficiencies of the *fum1* primers for different fumonisin producing strains, five different isolates belonging to three different *Fusarium* spp. were analyzed (*F. proliferatum 23*, *F. proliferatum 353*, *F. proliferatum 2763*, *F. verticillioides*, *F. nygamai*). These isolates were provided by the Vienna University of Technology and were internally numbered. The qPCR reagent concentrations and conditions were the same as mentioned above.

To evaluate the sensitivity of the assay 5 ng of purified genomic DNA from *F. graminearum* and *F. verticillioides* were serially diluted by a factor of five with maize DNA (50 ng/µl) to a concentration of 4.10 x 10^{-9} ng/µl. This was done for the singleplex as well as for the multiplex method.

The infected maize samples were divided into two sub-samples. Each sub-sample was extracted and the DNA was precipitated twice. Each precipitate was quantified twice with the three singleplex assays as well as with the newly developed multiplex qPCR method to ensure the repeatability of all parts of the method.

2.5 Determinaton of *Fusarium* toxins by LC-MS/MS

All maize samples were analyzed for the presence and concentrations of *Fusarium* metabolites by LC-MS/MS according to Malachová et al.[33]. The analytical method has been extended to cover 320 metabolites, transferred to a more sensitive mass spectrometer. In brief, 5 g of sample was weighed into a 50 ml polypropylene tube (Sarstedt, Nümbrecht, DE) and extracted with 20 ml acetonitrile/water/acetic acid (79:20:1, v/v/v) for 90 min on a GFL 3017 rotary shaker (GFL, Burgwedel, DE). The extracts were diluted in extraction solvent (ratio 1:1) and directly injected into the LC-MS/MS instrument.

3 Results and discussion

3.1 Development of a quantitative triplex PCR assay

During multiplex qPCR optimization 81 combinations of different annealing temperatures and times have been evaluated together with three concentrations of oligonucleotides (0.1 pM/µl, 0.05 pM/µl and 0.025 pM/µl). For the oligonucleotides adh1_fw, adh1_rev, adh1_probe and tri5_probe a concentration of 0.05 pM/µl was used. All other primers and probes were used at a concentration of 0.1 pM/µl. A gradient PCR from 52 °C to 60 °C was performed with these primers and the optimal thermal cycling conditions turned out to be 1 min 50 s at 95 °C, followed by 45 cycles of 15 s at 95 °C and 45 s at 58 °C. The multiplex assay worked most efficiently with a two-step protocol.

3.2 Specificity and sensitivity of the multiplex qPCR assay

The specificity of the *tri5* assay has previously been demonstrated by Brunner et al.[28] for wheat samples. All tested trichothecene producing strains were amplified with an efficiency of 0.91 ± 0.41. Waalwijk et al.[26] evaluated the specificity of the *fum1* assay and showed that no amplicons were generated from any of the non-fumonisin producers such as *F. equiseti*, *F. graminearum*, *F. oxysporum*, *F. semitectum* and *F. subglutinans*. The specificity of the *fum1* assay was also tested in this study using five different isolates from three different fumonisin producing *Fusarium* species, obtained and internally labelled by the Vienna University of Technology (*F. proliferatum* 23, *F. proliferatum* 353, *F. proliferatum* 2763, *F. verticillioides* and *F. nygamai*). The amplification efficiency for all tested strains was found to be 0.88 ± 0.43. All isolates scored positive for the *fum1* product, suggesting that the qPCR reaction is neither influenced by different isolates nor by species. Furthermore, no cross-reactivity was shown between fumonisin and trichothecene producing strains, which confirms that the primers and probes are group specific for either fumonisin or trichothecene producers (data shown in Table 1).

To determine the minimum amount of fungal template DNA necessary for quantification and the sensitivity of the assay, the multiplex qPCR method was carried out using dilution series of a mixture of pure fungal genomic DNA from *F. graminearum* and *F. verticillioides* in maize background DNA with a starting concentration of 5 ng/µl. The genomic DNA was serially diluted by a factor of 5 and used as a template. Due to high standard deviations for low target concentrations no more than 0.32 pg of target DNA per µl are quantifiable for both

Fusarium strains. The limit of quantification is defined as the minimum target concentration that remained within the linear regression line (Fig. 1). Lower concentrations could lead to false positive results. Considering a genome size of 41.7 Mb for *F. verticillioides* and 36.2 Mb for *F. graminearum*[34] this represents approximately seven or eight genome equivalents, respectively, calculated according to Staroscik[35]. Finally, no loss of sensitivity compared to the singleplex assays was observed when the multiplex method was applied. All assays were run in combination with the maize *adh1* gene for normalization.

Table 1 Specificity of the multiplex real-time PCR method against various fungal species

Fungal species	*fum1* detected	*tri5* detected
Fusarium proliferatum 23	+	-
Fusarium proliferatum 353	+	-
Fusarium proliferatum 2763	+	-
Fusarium verticillioides	+	-
Fusarium nygamai	+	-
Fusarium graminearum	-	+
Fusarium culmorum	-	+

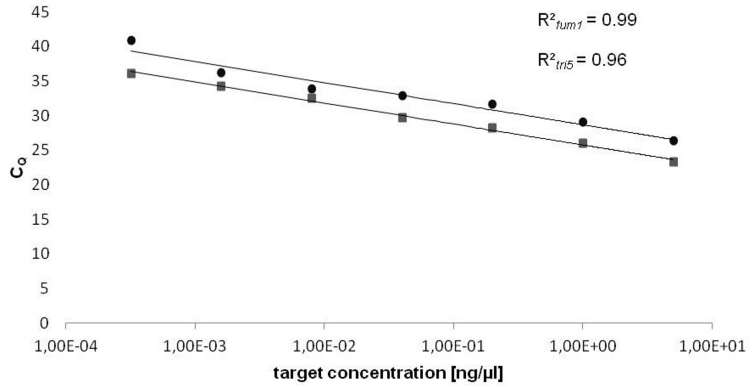

Fig. 1 Sensitivity of the multiplex real-time PCR assays. ● shows the serial dilution of *F. graminearum* and ■ shows *F. verticillioides* as target DNA. Both dilution series had a starting concentration of 5 ng and have been diluted by a factor of 5. Following dilutions were used: 5 ng, 1 ng, 200 pg, 40 pg, 8 pg, 1.6 pg, 0.32 pg

3.3 Reproducibility test of the multiplex qPCR assay

To determine whether the instrument used for quantification or the operator affect the results, reproducibility tests were performed by conducting all qPCR assays on two PCR thermal cyclers from different manufacturers. Furthermore, two operators prepared the qPCR assays. Comparing the results obtained by multiplex qPCR as well as the qPCR efficiencies, it could be shown that neither the instrument nor the operator had a significant influence on the obtained results.

3.4 Evaluation of the multiplex qPCR assay with *Fusarium* infested maize samples

The calculation of the starting concentrations in qPCR analysis requires the setting of a fluorescence threshold and the determination of the quantification cycle (C_Q) value, which is the fractional cycle number that is required to reach this threshold. The C_Q values are inversely proportional to the amount of initial target DNA in the sample. To quantify unknown amount of target DNA in the samples, appropriate DNA standards with known concentrations are needed. To obtain these standards, that optimally reflect the natural conditions, *Fusarium* free maize DNA was used as background and then spiked with varying amounts of *F. graminearum* or *F. verticillioides* DNA. From both DNA standards a dilution series was made by diluting them ten-fold with maize DNA to reduce the amount of *Fusarium* DNA in a constant maize background DNA concentration. Besides the quantification of the two different *Fusarium* DNAs, the maize specific gene *adh1*, encoding for an alcohol dehydrogenase, was included as a reference to normalize variations in DNA-extraction yields. A similar strategy is applied by the European Reference Laboratories for GM Food & Feed for the determination of the relative content of GM events in the total maize DNA.[29]

Table 2 shows the C_Q values of the serial diluted DNA standards as well as the efficiencies of the multiplex assay. The efficiency of the assay is derived from the standard curve and is calculated according to formula 1:

$$Efficiency = dilution\ factor^{\left(\frac{-1}{slope}\right)} - 1$$

The slope for the calculation of the efficiency is obtained by the linear regression line of the standard curve. The calculated efficiencies were 0.94, 0.98 and 1.03 for the *tri5*, *fum1* and *adh1* target fragments, respectively.

Table 2 C_Q values of serial diluted DNA standards and efficiencies for the three targets quantified simultaneously by the multiplex PCR

Concentration of fungal DNA (*fum1* or *tri5*)	**C_Q for trichothecene producing isolates**	**C_Q for fumonisin producing isolates**	**Concentration of maize DNA standard**	**C_Q for maize DNA amount**
5 ng/µl	26.86 (± 0.23)	23.47 (± 0.21)	50 ng/µl	24.25 (± 0.23)
0.5 ng/µl	30.50 (± 0.43)	26.73 (± 0.17)	25 ng/µl	25.13 (± 0.12)
0.05 ng/µl	33.80 (± 0.30)	30.20 (± 0.53)	12.5 ng/µl	26.10 (± 0.28)
			6.25 ng/µl	27.18 (± 0.41)
Efficiency	0.94	0.98	Efficiency	1.03
R^2	0.9992	0.9997	R^2	0.9979

To get a deeper insight into the repeatability of the whole analysis procedure, including the various steps of DNA extraction and quantification by three singleplex PCR assays and by the here developed triplex test, twenty-four maize field samples were collected from four different locations in Austria (locations A, B, C and D) and tested for the presence of *Fusarium* DNA. To identify the steps having the highest influence on the total error of the method, all samples were divided into two sub-samples. Each sub-sample was used for DNA isolation and the DNA was finally precipitated twice, as this can be considered as the most crucial step of the extraction procedure. Moreover, every precipitate of a sample was analyzed two times, either with the three separate singleplex assays or the multiplex qPCR method (Fig. 2).

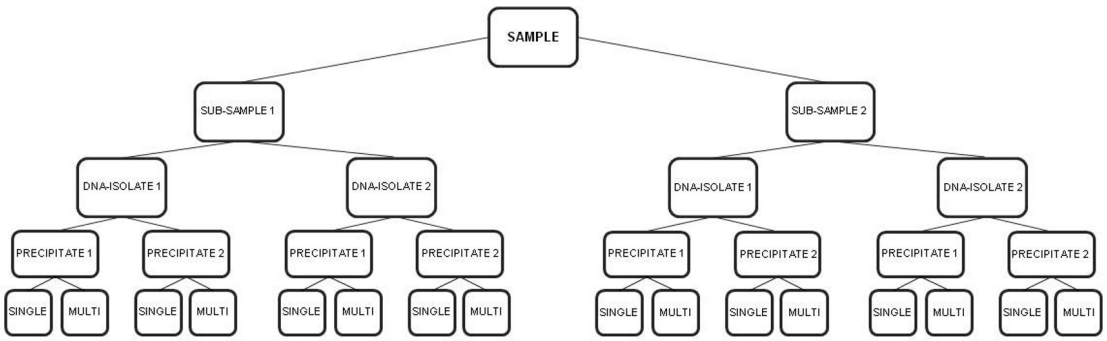

Fig. 2 Scheme of sample analyses to evaluate the repeatability of the crucial steps of the analysis procedure. Every sample was divided into two sub-samples which were then used for DNA isolation. Each DNA isolate was finally precipitated twice and analyzed two times, either with the three singleplex assays or the multiplex method.

The results show low variations between the two sub-samples, the individual precipitates and no significant differences result from the PCR analysis. In general, a mean total error of 23 % occurs over the whole analysis procedure. Going more into detail 5 % of the observed error are caused by dividing the sample into two sub-samples, 10 % arise from the DNA precipitation step and 8 % of the error results from the multiplex qPCR method. For the calculation of the error rates 48 values for sub-sample extraction, 96 values for precipitation and 192 values for qPCR analysis were included for each gene *(adh1*, *fum1* and *tri5*).

Finally, the infection of the samples was calculated based on formula 2 previously published by Brunner et al.[28] referring the fungal DNA to the total extracted DNA from a sample,

$$\text{Infection [\%]} = \frac{Fusarium\ \text{DNA [ng]}}{\text{total DNA [ng]}} \text{ x } 100$$

where "*Fusarium* DNA" is the DNA from trichothecene or fumonisin producing *Fusarium* species and "total DNA" is the sum of DNA from trichothecene producing *Fusarium* species, fumonisin producing *Fusarium* species and maize DNA.

The analyzed samples show a wide range of *Fusarium* infection, both for fumonisin and for trichothecene producing species. All analyzed samples were positive for *fum1* DNA and *tri5* DNA. Fig. 3 shows that comparable results were obtained with three singleplex assays and the rapid multiplex test. The grade of observed infection with fumonisin producing *Fusarium* species varies between 8.2×10^{-4} ‰ and 0.22 ‰, whereas the range of infection with *tri5* producing strains was much higher and ranged from 0.10 % to 3.33 %. This huge difference of infection between the two groups might be due to that fact that fumonisin producing strains commonly appear in southern regions. In cooler regions like Austria, fumonisin production is reduced and toxins produced by other *Fusarium* species become more important and more prevalent.[11] The results for the fumonisin producing group inherently show a higher standard deviation due to the very low amount of DNA detected in all samples. The mean relative standard deviation (RSD*)* for the samples for trichothecene producing species is 7.17 % for the singleplex, and 22.71 % for the multiplex method. For the *fum1* assay the mean RSD shows 17.19 % for the singleplex and 23.30 % for the multiplex method. All RSD values are referred to the complete analysis method, including DNA isolation and the qPCR analysis. The loss of precision of the triplex *tri5* multiplex in contrast to the singleplex method might be attributable to the many oligonucleotides in the multiplex assay interacting among them. A slight loss of precision was therefore expectable and is negligible due to the considerable correlations of the results between the two methods. Furthermore, the costs and analysis time are reduced by a factor of three for the triplex method.

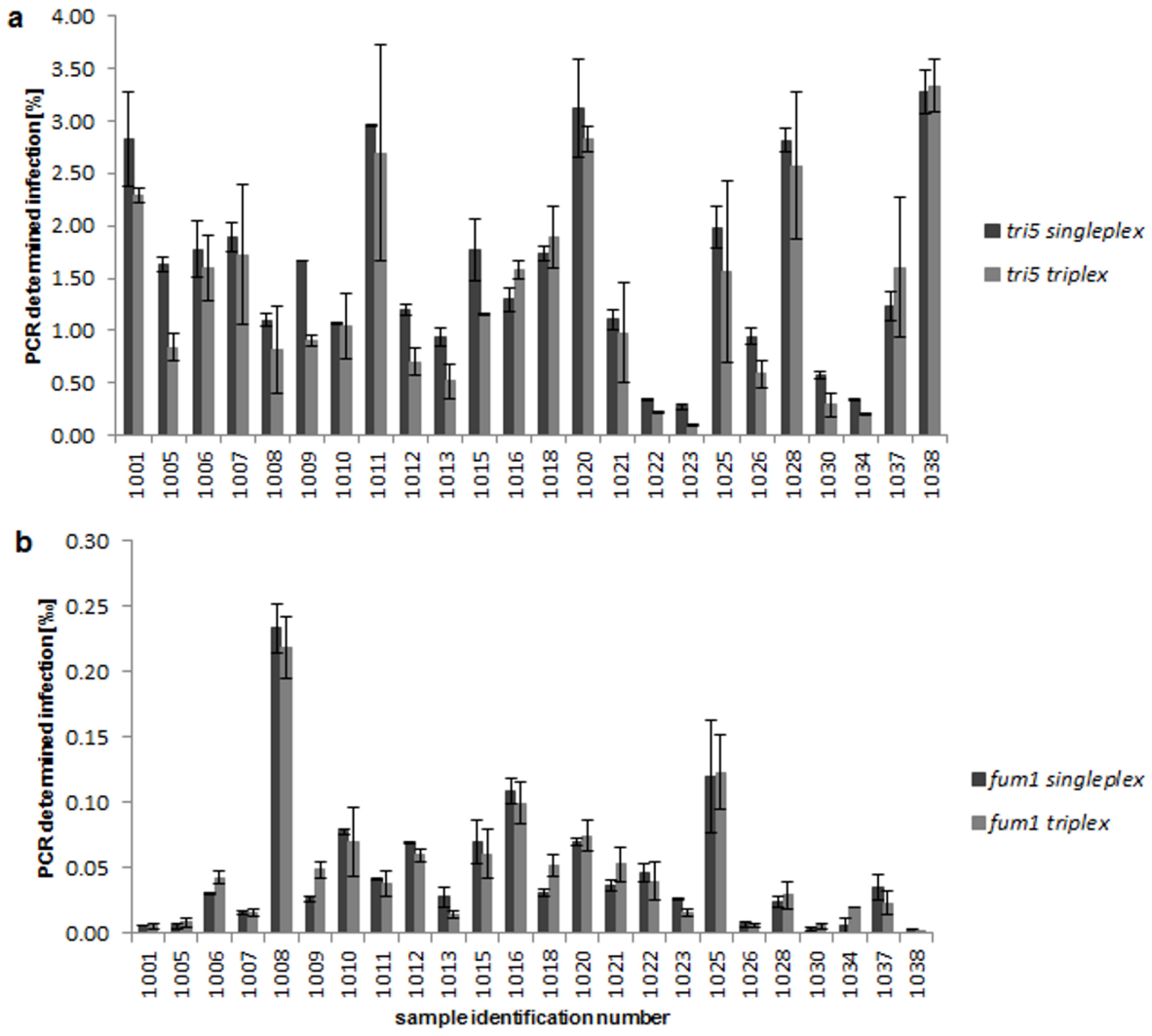

Fig. 3 Analysis of Austrian field samples. The bars show the results either obtained by the singleplex (dark grey) or the newly developed multiplex (light grey) method. **a)** PCR-determined infection of maize samples with trichothecene producing species, **b)** Infection of samples with fumonisin producing *Fusarium* species

3.5 Comparison of LC-MS/MS determined mycotoxin concentrations with the real-time PCR determined *Fusarium* infection

The twenty-four previously extracted maize samples were now used to analyze whether the qPCR results for *Fusarium* DNA correlate either with the trichothecene or the fumonisin content. Therefore, the mycotoxin concentration of each sample was determined by LC-MS/MS. Five g sample were extracted with 20 ml acetonitrile/water/acetic acid (79:20:1, v/v/v) and analyzed according to Malachová et al.[33] The qPCR determined infections observed with the multiplex method were plotted against the mycotoxin concentrations (Fig. 4).

The PCR primers for the *fum1* and *tri5* gene quantification are specific for either all *Fusarium* species producing toxins belonging to the class of fumonisins or the *Fusarium* species producing metabolites belonging to the group of the trichothecenes, respectively. For this reason, the *tri5* infection, was not only compared to the prevalent DON concentration but to the total amount of trichothecenes including DON-3-glucoside (resulting from *in planta* metabolisation of DON), NIV, 3-ADON and also the type A trichothecenes DAS, T-2, HT-2 and NEO.[36] It was found that the PCR results correlate slightly better with the sum of the eight toxins than with DON only. For the comparison of the *fum1* infection with the fumonisin concentrations, the toxins FB_1, FB_2 and FB_3 were used as a sum parameter. Fig. 4 shows that a certain correspondence between the infection of a maize sample calculated according to formula 2 and the toxin content determined by LC-MS/MS can be observed. The

coefficient of determination was found to be $R^2 = 0.64$ for the correlation of the trichothecene content and the PCR determined infection. Approximately the same R^2 was observed for the fumonisin concentrations and the infection determined by qPCR.

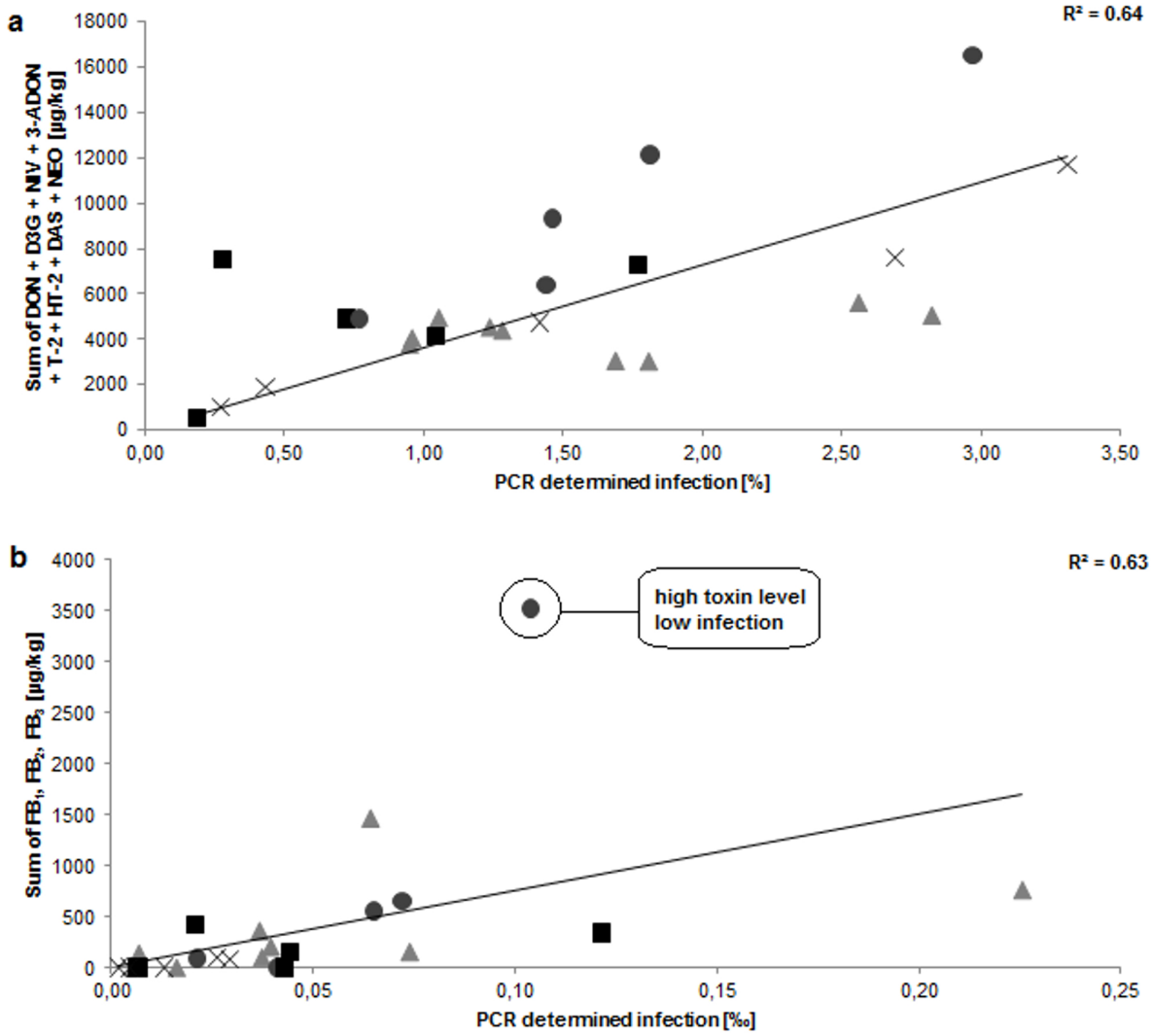

Fig. 4 Correlation of mycotoxin concentrations and PCR determined infection rates. The four different locations are labeled as follows: ■A, ●B, ▲C and ✕D **a)** Results for trichothecene producing strains; the type B trichothecenes DON, D3G, NIV and 3-ADON as well as the type A trichothecenes T-2, HT-2, DAS and NEO were used as a sum parameter **b)** Correlation of the infection with fumonisin producing strains with the fumonisins FB_1, FB_2 and FB_3

Although a certain correlation between the mycotoxin content of a sample and the infection determined by qPCR can be observed, it is obvious that *Fusarium* isolates present at location B produce presumably more toxin per fungal biomass, whereas at location C the situation is converse. This fact demonstrates that the infection determined by qPCR analysis provides additional information which might be relevant for the determination of the resistance of new crop lines in breeding programs.

In general, two methods are applied for the registrations of new crop varieties: visual scoring and mycotoxin analysis. But these methods only indirectly determine the resistance of plants which is defined as the pathogen growth on or in the plant. Previous experiments stated that the amount of fungal mycelia formed during infection not always correlates well with disease symptoms[28, 37] and also the amount of accumulated DON does not necessarily reflect differences in resistance. Genotypes with good resistance based on visual scoring might show high or low toxin contaminations. Furthermore, highly resistant wheat varieties can often not be classified by visual scoring anymore as the infection proceeds without any symptoms but nevertheless leads to high toxin accumulation[25]. The supreme sensitivity of qPCR registers even minor amounts of fungal biomass[17].

4 Conclusion

In the last decade, several studies describe two distinct approaches for *Fusarium* quantification, species specific assays which quantify individual *Fusarium* species or group specific assays which measure the abundance of a gene owned by a group of different species sharing a common biosynthetic pathway (e.g. for mycotoxin production).[26, 28, 38] Two group specific quantitative PCR assays were used frequently throughout the last decade: one test which quantifies all trichothecene producing *Fusarium* species[23] and another one for all fumonisin producing species.[19] However, until now these tests were only available as two quantitative singleplex tests or as a duplex assay[25] which is only qualitative.

In this study we developed for the first time a multiplex qPCR assay which allows the screening of maize samples for all *Fusarium* species producing the most relevant mycotoxins in maize, trichothecenes and fumonisins. Besides the two fungal toxin genes, *tri5* and *fum1*, the maize gene *adh1* is used additionally as a reference gene to compensate for varying DNA extraction yields, similar to the established system for GMO analysis.[26, 27] The newly developed multiplex qPCR method is a high-throughput, reliable diagnostic tool that allows rapid screening of high sample numbers in a short time. This assay was designed to minimize costs by reducing the analysis time by a factor of more than 60%. This assay provides a perfect complementation to mycotoxin analysis to facilitate the classification of the resistance of new crop lines in breeding programs.

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