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EXPERIMENTAL ARTICLES

Microbiological and Sybr® Green Real-Time PCR Detection of Major *Fusarium* Head Blight Pathogens on Wheat Ears[1]

M. Moradi[a, 2], E.-C. Oerke[a], U. Steiner[a], D. Tesfaye[b], K. Schellander[b], and H.-W. Dehne[a]

[a] *Institute of Crop Science and Resource Conservation (INRES)—Phytomedicine, Rheinische Friedrich-Wilhelms-Universität Bonn, Nussallee 9, 53115 Bonn, Germany*

[b] *Institute of Animal Science, Rheinische Friedrich-Wilhelms-Universität Bonn, Endenicher Allee 15, 53115 Bonn, Germany*

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Abstract—*Fusarium* head blight (FHB) caused by several *Fusarium* species is one of the most serious diseases affecting wheat throughout the world. The efficiency of microbiological assays and real-time PCR to quantify major FHB pathogens in wheat ears after inoculation with *F. graminearum, F. culmorum, F. avenaceum* and *F. poae* under greenhouse and field conditions were evaluated. The frequency of infected kernel, content of fungal biomass, disease severity and kernel weight were determined. To measure the fungal biomass an improved DNA extraction method and a Sybr® Green real-time PCR were developed. The Sybr® Green real-time PCR proved to be highly specific for individual detection of the species in a matrix including fungal and plant DNA. The effect of *Fusarium* infection on visible FHB severity, frequency of infected kernels and thousand-kernel mass (TKM) significantly depended on the *Fusarium* species/isolate. *F. graminearum* resulted in highest disease level, frequency of infected kernels, content of fungal biomass, and TKM reduction followed by *F. culmorum, F. avenaceum* and *F. poae*, respectively. The comparison of frequency and intensity of kernel colonization proved differences in aggressiveness and development of the fungi in the kernels. Only for *F. graminearum*, the most aggressive isolate, application of microbiological and real-time PCR assays gave similar results. For the other species, the intensity of kernel colonization was lower than expected from the frequency of infection.

Key words: real-time PCR, Fusarium head blight, quantification, *Fusarium graminearum*, aggressiveness

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Fusarium head blight (FHB) of small grains was first described more than a century ago and was considered a major threat to wheat and barley during the early years of the last century. In recent years, FHB has again increased worldwide with great concerns arising from mycotoxin contamination and increase in the risk of human and animal health [1]. Head blight is caused by several *Fusarium* species; distribution and predominance of species significantly vary among climatic conditions, geographical zones, countries, and years [2–4]. Current methods such as visual assessment of disease severity, infected ears or spikelets and damaged or infected kernels estimate pathogen populations indirectly [5, 6]. Attempts to evaluate the frequency of fungal species in a particular context, in the absence of selective media is confound, because the relative amount of each pathogen may not be determined accurately [7]. Morphological identification of *Fusarium* species showing similarities with other species makes the task difficult. For example, *F. avenaceum* and *F. arthrosporioides* are very difficult to separate by the morphological characteristics of their spores [8]. Using species-specific primers based on RAPD-PCR, UP-PCR and positive/negative classical PCR, it is possible to separate most of the *F. arthrosporioides* isolates from the closely related *F. avenaceum* isolates [8]. On the other hand, *F. avenaceum* and *F. tricinctum* had a close genetic relationship using molecular markers in spite of belonging to different morphological taxonomic sections (*Roseum* and *Sporotrichiella*, respectively). However, RAPD-based phylogenetic profiling revealed interspecies differences large enough to distinguish clearly between isolates of the two species [9].

Recent advances in molecular systematic of fungi provide extensive DNA sequence information that is of great benefit in molecular detection and diagnostics, especially for large genera whose species have similar morphological characteristics (e.g. *Fusarium* and *Pythium*) [10]. PCR assays based on species-specific primers offer accurate, rapid identification and quantification for the major FHB pathogens in different tissues and plant matrixes in singleplex or multiplex PCR, simultaneously [7, 11]. Many species-specific primers for detection *Fusarium* species have been designed [3, 7–9, 12–14].

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[2] Corresponding author; e-mail: moradi@pri.ir

Recently, real-time PCR using internal labelled probes have been developed for the major FHB pathogens in different plant matrices [11, 13–15]. Real-time PCR is a technique of collecting data throughout the PCR process as it occurs, thus combining amplification and detection into a single step [16] which takes less than 1 day compared to 2–3 weeks required for microbiological detection. Theoretically, there is a quantitative relationship between the amount of starting material and the PCR product at any cycle of the PCR [17]. This indicates that PCR may allow the quantification of fungal DNA. Various real-time detection systems can be divided into two approaches based on nonspecific double-stranded DNA intercalator dyes like SYBR® Green I and internal probes labeled with different reporter dyes which are applicable for quantification [13, 18, 19].

The objectives of the present study were I) to develop a real-time PCR with SYBR® Green I for the quantification of major FHB pathogens in wheat kernels as compared to microbiological assessments and II) to evaluate differences between *Fusarium* species/isolates in aggressiveness and development in the kernels.

MATERIALS AND METHODS

Fungi. Fungal species including isolates of *Fusarium graminearum, F. culmorum, F. avenaceum, F. poae, F. langsethiae, F. tricinctum, F. sporotrichioides, F. cerealis, Blumeria graminis, Puccinia triticina, Alternaria* sp., *Trichoderma* sp., *Aspergillus* sp. and *Penicillium* sp. commonly associated with FHB and other wheat diseases were used. All isolates were from the collection of the Institute of Crop Science and Resource Conservation, University of Bonn, Germany, except for *F. poae* (DSM 62376) which was from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

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

A field trial was conducted at Poppelsdorf experimental station, University of Bonn, Germany in 2006. Spring wheat cv. Taifun was sown in April and sprayed with Mospilan SG® (acetamiprid, Nisso Chemicals Europe, Duesseldorf, Germany) to control aphids. The plots were separately harvested using a combine harvester on July 24, 2006. The kernels were stored at –20°C for further analysis.

Inoculum production. Single hypha isolates of *Fusarium* species were used in the experiments: FA 1.7 (*F. ave-naceum*, isolated at Dormagen in 1995), FC 3.11 (*F. culmorum*; Lage-Ohrsen, 2002), FG 5.1 (*F. graminearum*; Blankenheim, 1996), and FP 7.8 (*F. poae*, Lage-Ohrsen, 2002) from winter wheat fields in North Rhine-Westphalia, Germany.

In 2005, *Fusarium* spp. were grown on potato dextrose agar (PDA 12.5 g/l, agar-agar 19.5 g/l, Merck, Darmstadt Germany) for 21 days under UV light. In 2006, *Fusarium* spp. were cultured in potato-dextrose broth (24 g/l) in 1000-ml Erlenmeyer flasks for 2-5 days in darkness on a shaker (200 rpm) at room temperature. 500 μl of suspension were plated onto potato dextrose agar. Plates were incubated in darkness up to 3 days. Conidia were harvested by washing the mycelium with sterile distilled water and lightly scraping with spatula to dislodge the conidia. The suspension was passed through double layered cheese-cloth.

Inoculation of wheat ears. At mid flowering (GS 65) wheat ears were inoculated with single isolates of *Fusarium* species with concentrations of 5×10^4 conidia ml^{-1}. Control plants were sprayed with sterile distilled water in the same way. After inoculation, pots or plots were covered with plastic bags for 48 hours to ensure high relative humidity for infection. The experiments were conducted in a completely randomized experimental design with 5 replications. In all experiments, every wheat ear was sprayed with 2 ml of inoculum or sterile distilled water.

Parameters of Fungal Development

Visual disease ratings. Disease severity was assessed as percentage of bleached spikelets 14, 21 and 28 days after inoculation using a nine-class rating scale [20] in which 1 = no infection, 2 = <5; 3 = 5–15; 4 = 16–25%; 5 = 26–45%; 6 = 46–65%; 7 = 66–85%; 8 = 86–95%, and 9 = 96—100% of bleached spikelets. The mean values of the three disease severity ratings were determined.

Microbiological assessment of *Fusarium*-infected kernels. For determining the frequency of infected kernels and the amount of fungal DNA in wheat kernels, 50 kernels per replicate were cut into two pieces perpendicular to the length (one piece for microbiological, the other one for real-time PCR). Czapek-Dox-Iprodione-Dicloran agar (CZID) [21] was used for re-isolation of *Fusarium*pp. Synthetic nutrient-poor mineral agar (SNA) [22] and Banana leaf agar (BLA) [23] were used to grow the isolates for morphological identification [24].

Yield assessment. In greenhouse experiments, kernel yield was recorded for every replicate. Data are shown based on 1000-kernel mass which was calculated from kernel yield and number of kernels per replicate. In field experiment, data were determined based on four times calculating kernel mass for 1000 kernels per replicate.

Table 1. Characteristics of species-specific primers for detecting *Fusarium* species in wheat kernels

Primer name	Target species	Sequence of primer	Annealing temperature	Product size (bp)	Reference
MGB-GRA	*F. graminearum*	GGCGCTTCTCGTGAACACA TGGCTAAACAGCACGAATGC	55°C	94	[10]
MGB-CUL	*F. culmorum*	TCACCCAAGACGGGAATGA GAACGCTGCCCTCAAGCTT	55°C	60	[10]
MGB-AVE	*F. avenaceum*	CAAGCCCACAGACACGTTGT CCATCGCCGTGGCTTTC	57°C	58	[10]
Fp82	*F. poae*	CAAGCAAACAGGCTCTTCACC TGTTCCACCTCAGTGACAGGTT	60°C	220	[9]

Molecular Quantification of Fusarium DNA

DNA extraction. Twenty ml of cetyltrimethylammonium bromide (CTAB)-extraction buffer (10 mM Tris, 20 mM EDTA, 0.02 M CTAB, 0.8 M NaCl, 0.03 M N-laurylsarcosine, 0.13 M sorbitol, 1% (w/v) polyvinylpolypyrolidone, pH set to 8.0 with NaOH), 40 µl mercaptoethanol and 50 µl proteinase K (from a stock solution 10 mg/ml), were added to 250 mg ground grains in 50-ml centrifugation tube and mixed vigorously [25]. The mixture was incubated at 65°C for 60 min and mixed after every 10 min. Eight hundred µl of the upper phase was transferred to a 2 ml new tube containing 5 µl of RNAase (50 mg/ml) and incubated for 10 min at 65°C. Eight hundreds µl of chloroform-isoamyl alcohol (24:1) was added into each tube. The samples were mixed by inverting the tubes and centrifuged for 10 min at 5.000 *g* at room temperature. The aqueous phase was transferred into a 1.5 ml tube containing 500 µl isopropanol, mixed and incubated for 20 min at room temperature and centrifuged for 15 min at 15000 *g* at room temperature. The pellet was washed with 70% (v/v) ethanol, dried and dissolved in 200 µl TE buffer and incubated at 4°C over night and then at –20°C.

Tests on primer specificity and sensitivity. Species-specific primers for *F. graminearum*, *F. culmorum*, *F. avenaceum* [13] and *F. poae* [12] were used to amplify specific fragment using classical PCR (Thermocycler BIO RAD iCycler BIO RAD, München) (Table 1). All primers were synthesized by Carl Roth Company (Karlsruhe, Germany). DNA of fungal species was extracted from 50–100 mg fresh mycelia grown on agar media using the Plant DNeasy Mini Kit (Qiagen, Hilden, Germany). The PCR products were loaded on 2% (w/v) agarose gel and run in 1 × TAE buffer by staining with ethidium bromide.

To confirm the specificity of primers, PCR products were purified using ExoSAP-IT. A mixture of 1 µl of ExoSAP-IT with 5 µl of PCR product was incubated at 37°C for 30 min followed by ExoSAP-IT inactivation at 80°C for 15 min. The pure DNA template was sequenced using SEQ 8000 Genetic Analysis System (Beckman coulter, Krefeld, Germany) using CEQ Dye Terminator Cycle sequencing (DTCS) Quick start kit. The sequencing PCR reaction was done according to the recommendation of the company. The sequence of the fragments was utilized to confirm the specificity of primers using available information in data bank (http://www.ncbi.nlm.nih.gov/BLAST/).

Real-time PCR. Real-time PCR reactions were performed in an ABI Prism® 7000 SDS (Applied Biosystems, Foster City, USA) and SYBR® Green was used as a double-strand DNA-specific fluorescent dye. Prior to quantification, primer optimisation was carried out for both forward and reverse primers in presence of template (DNA) and non-template as control to avoid primer dimer formation. The lowest threshold cycle without primer dimer formation was used to perform subsequent real-time PCRs. Standard curves were generated using a serial dilution (0.9, 9, 90, 900 and 9000 pg) of purified genomic DNA of *Fusarium* spp. DNA was extracted from the mycelium of *Fusarium* spp. as described for wheat kernel previously. Further chloroform-isoamyl alcohol and ammonium acetate precipitation steps were carried out to obtain high quality DNA. Polymerase chain reactions were carried out in 20 µl reaction volume containing 10 µl SYBR® Green Jump start TM Taq Ready MixbTM (Sigma, Germany), 0.2 µl Rox as internal reference dye (Sigma, Germany), 0.4 µM of each forward and reverse primers, 2 µl genomic DNA. PCR reactions were performed in duplicates for standard curves and samples to control the reproducibility of quantitative results. A universal thermal cycling protocol (10 s at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C) was used for the quantification. The specificity of real-time PCR during quantification was verified by melting curve analyses. The reproducibility of experiments was tested on 250 mg aliquots of a positive sample for *F. graminearum*, *F. cul-*

morum, *F. avenaceum* and *F. poae* using real-time PCR assays as described by Waalwijk et al. [10].

Statistical analysis. Means of disease severity ratings, 1000-kernel mass, frequency of re-isolation, and the content of fungal DNA were determined for each replication. The effects of *Fusarium* species on these parameters were analysed using proc GLM procedures (SAS Vers. 9.0, SAS Institute, Inc., Cary, NC). Means were compared using Duncan's new multiple range test at 5% probability. Data were log-transferred prior to analysis when necessary.

RESULTS

Specificity of Primers

PCR was performed with the species-specific primer pairs resulted in single fragments for individual *Fusarium* species. The Fp82F/R, MGB-GRA, MGB-CUL and MGB-AVE primers amplified fragments with the size of 220, 100, 60 and 58 bp that were unique for all isolates of *F. poae*, *F. graminearum*, *F. culmorum* and *F. avenaceum*, respectively. No cross reactivity was observed with other fungal DNA (*Blumeria graminis*, P*uccinia triticina*, *Trichoderma* sp., *Alternaria* sp., *Aspergillus* sp., *Penicillium* sp.) and matrix DNA as well as in the negative control (no DNA template; Fig. 1.1–1.4), indicating the specificity of the primer sets under these experimental conditions.

In some cases, MGB-GRA, MGB-CUL primers amplified a PCR product with matrix plant DNA (e.g. Fig. 1.3). Further assays showed that when the annealing temperature increased to 60°C, the primers gave highly specific amplification. In most cases the results of morphological identification of tested *Fusarium* species were confirmed using PCR assays with species-specific primers for individual species. However, there was no amplification in three isolates (7.2, 7.10 and 7.13) of *F. poae*, with primer Fp82 F/R (Fig. 1.1).

SYBR® Green Real-Time PCR for the Quantification of Fusarium Species from Wheat Ears

Melting curve analysis. The results of real-time PCR using SYBR® Green I for *Fusarium graminearum*, *F. culmorum*, *F. avenaceum* and *F. poae* confirmed the results of the primer specificity obtained with conventional PCR. Using the species-specific primers MGB-GRA, MGB-CUL, MGB-AVE, and Fp82 no PCR amplification or melting curve was observed neither for the negative control nor for other *Fusarium* species or other fungal species commonly associated with wheat (Fig. 2). Melting curve analysis revealed the presence of a single peak for individual *Fusarium* species. Melting temperatures (MT) for amplicons were 81.2, 77.5, 79.0 and 81.4°C for *F. graminearum, F. culmorum, F. avenaceum* and *F. poae*, respectively. The concentration of fungal DNA did not affect the melting temperature of the amplicons. There was a positive correlation between the amounts of PCR product and the height of the melting curves.

Table 2. Reproducibility of species-specific real-time PCR assays for the quantification of four *Fusarium* species in wheat kernels; threshold cycle values and standard deviation are given for 9 DNA preparations using CTAB method, intra-assays and inter-assays

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

Standard curve. For the quantification of *Fusarium* species via real-time PCR, threshold cycles correlated with a known amount of *Fusarium* DNA. For all *Fusarium* species standard curves were prepared based on ten threshold cycles from ten-fold serially diluted DNA in two replications of real-time PCR. Standard curves are presented in Fig. 3 for *F. graminearum, F. culmorum, F. avenaceum* and *F. poae*.

Overall, there were highly significant ($R^2 \geq 0.99$) negative correlations between threshold cycle values and the amounts of fungal DNA for all *Fusarium* species. The coefficient of determination ranged from 0.99 to 0.999 for *F. culmorum* and *F. graminearum*, 0.989 to 0.997 for *F. avenaceum* and 0.985 to 0.995 for *F. poae*. The standard deviation of the coefficients of correlations between threshold cycle values and amount of fungal DNA in different real-time PCR runs was less than 0.007 for the *Fusarium* species. The slope ranged from −3.32 to −3.75 and the amplification efficiency varied from 1.88 to 2.0.

Results of reproducibility of DNA extraction procedure, intra- and inter- assays have been summarized in Table 2. The standard deviation of threshold cycle values from nine DNA preparations, intra-assays and inter-assays ranged from 0.10 to 0.44, 0.10 to 0.15, 0.2 to 0.33 and 0.11 to 0.66 for *F. graminearum, F. culmorum, F. avenaceum* and *F. poae*, respectively. The standard deviation of threshold cycle values among DNA preparations was higher than that for the other assays and reached 0.66 for *F. poae*, while intra-assays had the lowest standard deviations. *Fusarium culmorum* had the lowest standard deviation of threshold cycle values in all assays.

Assessment of FHB Severity, Frequency of Infected Kernels and Fusarium Biomass

Results from experiments under greenhouse conditions and in the field demonstrated high

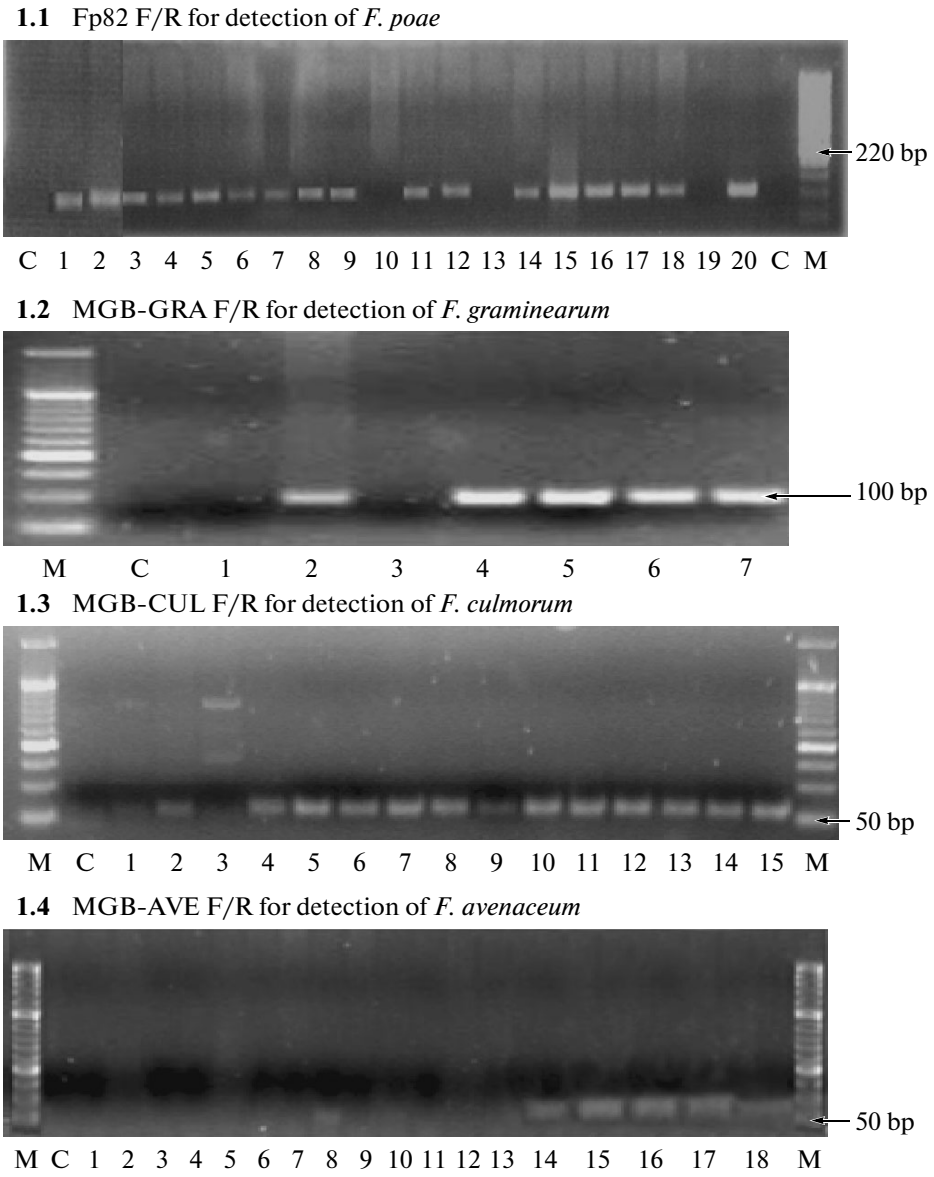

Fig. 1. Agarose gel electrophoresis of species-specific PCR products for the detection of *Fusarium* species:

Fig 1.1. PCR products from isolates of *F. poae* using primer pair Fp82 F/R. Lanes 1–6, *F. poae* 5.1, 2, 3, 4, 5 and 5.6; Lanes 7–20, *F. poae* 7.16, 15, 14, 13, 12, 11, 10, 9, 8, 6, 5, 3, 2 and 62376, respectively.

Fig. 1.2. PCR products from isolates of *F. graminearum* using primer pair MGB-GRA F/R. M, DNA ladder; C, non-template control (water); Lanes, 1–3 wheat stem, kernel and leaf; Lanes 4–7, *F. graminearum* 5.10, 15, 17 and 5.19 isolates, respectively.

Fig. 1.3. PCR products from isolates of *F. culmorum* using primer pair MGB-CUL F/R. M, DNA ladder; C, non-template control (water); Lanes 1–3, wheat stem, kernel and leaf; Lanes 4–15 *F. culmorum* 3.6, 9, 12, 14, 16, 17, 18, 22, 33, 35, 36 and 3.37 isolates, respectively.

Fig. 1.4. PCR products from isolates of *F. avenaceum* using primer pair MGB-AVE F/R. M, DNA ladder; C, non-template control (water); Lanes 1–3, wheat stem, kernel and leaf; Lanes 5–7, *Blumeria graminis*, *Puccinia triticina*, *Trichoderma* sp. and *Alternaria* sp.; Lanes 8–11, *F. tricinctum*, two isolates of *F. sporotrichioides* and *F. cerealis*; Lanes 12–13 *Aspergillus* sp. and *Penicillium* sp.; Lanes, 13–18 *F. avenaceum* 1–7, 8, 9, 12, and 1.16, respectively.

reproducibility of the differences among *Fusarium* isolates (Table 3). The *Fusarium* species/isolates significantly affected visible FHB severity. In all experiments, *F. graminearum* resulted in the highest disease level, highest frequency of infected kernels, highest fungal biomass in kernels and highest reduction of 1000-kernei mass followed by *F. culmorum, F. avenaceum* and *F. poae*, respectively. All species/isolates except *F poae* caused head blight symptoms and reduced kernel mass. *F. graminearum* reduced kernel mass by 21 to 50%; the effect of *F. culmorum* was significantly lower. Kernel mass of

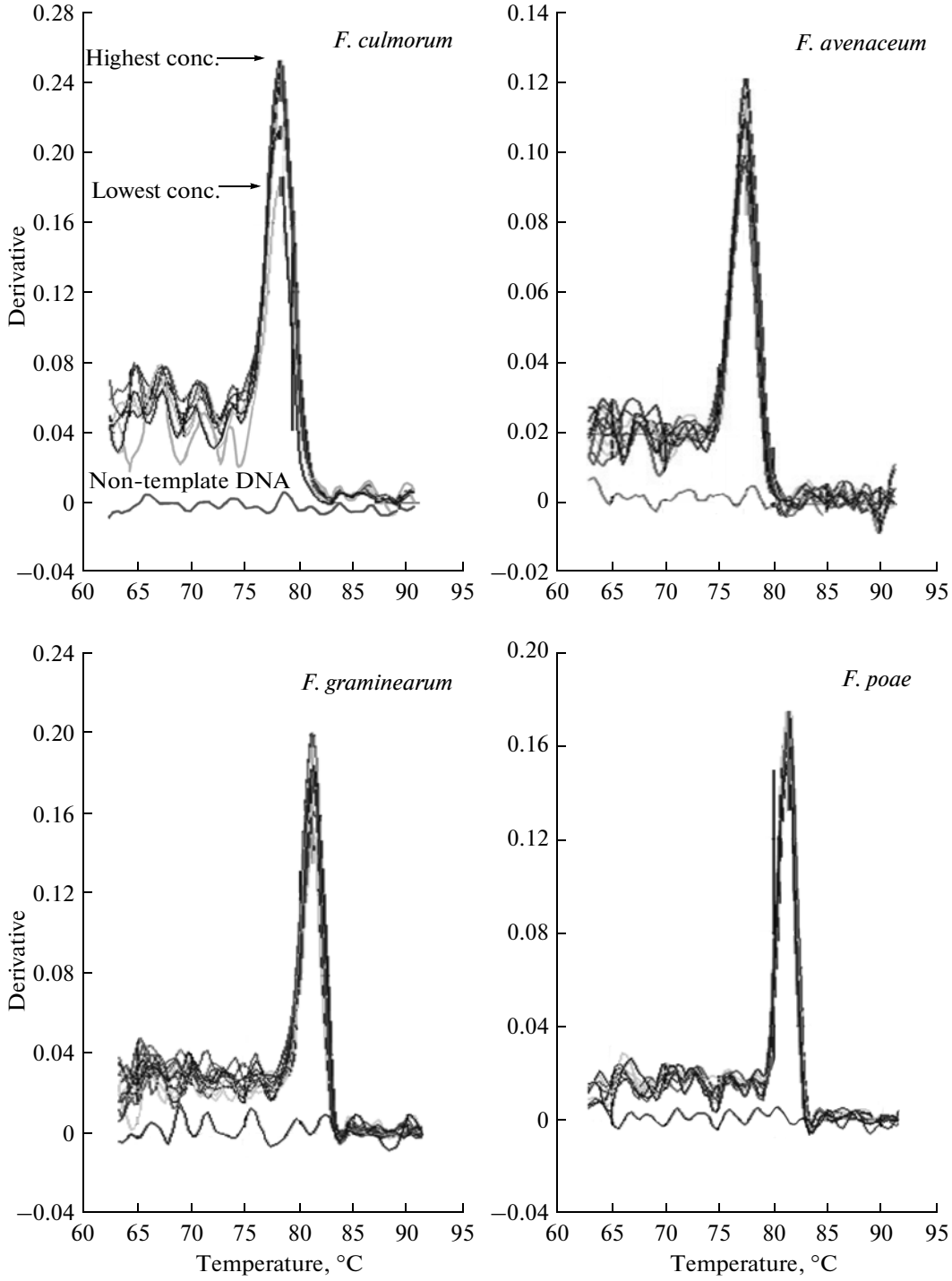

Fig. 2. DNA melting curves (plot of fluorescence derivative dF/dT versus temperature °C) of specific amplicons for *F. avenaceum, F. culmorum, F. graminearum* and *F. poae* in a matrix of fungal and plant DNA.

wheat inoculated with *F. avenaceum* and *F. poae* were not significantly different from those of non-inoculated plants. Inoculations with *F. graminearum* resulted by far in the highest amount of fungal DNA in kernels, followed by *F. culmorum, F. avenaceum* and *F. poae* with 54, 5, and 1%, respectively, of the fungal DNA as compared to *F. graminearum* in greenhouse and field experiments. Similarly, the frequency of kernels infected by *F. culmorum, F. avenaceum* and *F. poae*, reached 74, 40 and 7% of the frequency of *F. graminearum* infected kernels, respectively.

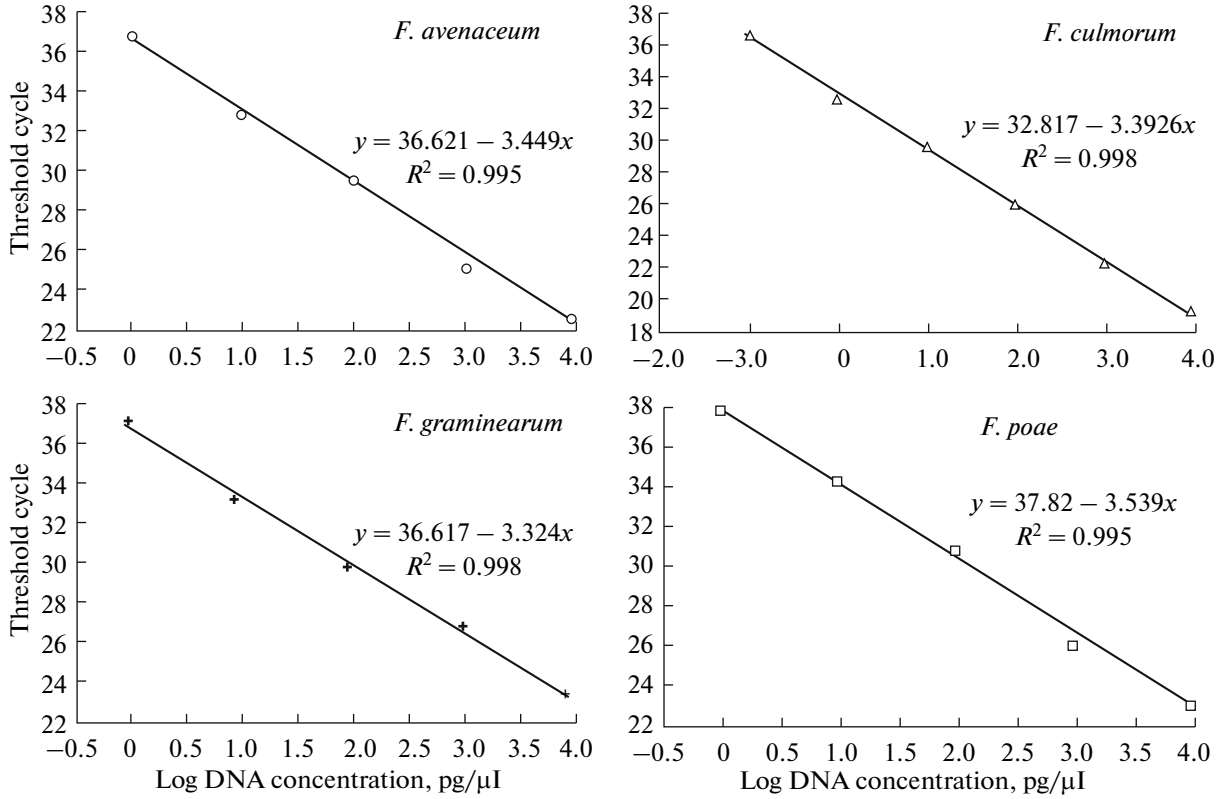

Fig. 3. Standard curves for the quantification of four *Fusarium* species using SYBR® Green real-time PCR.

DISCUSSION

The CTAB procedure for DNA extraction described here works well for extracting high quality DNA from various wheat tissue samples as well as from mycelium of *Fusarium* species. High reproducibility among replicates of the grain samples confirmed the reliability of this protocol for quantification of FHB pathogens using real-time PCR and confirmed results from Brandfass and Karlovsky [26] that increasing the amount of plant material reduces the sampling error and improves the reproducibility of DNA yield. In many other studies either on routine DNA extraction or comparison of different extraction methods, CTAB method has been included and proved as one of the best methods [27–29]. Sample sizes for DNA extraction described here were 250 mg to 1 g and gave high quantity and quality of DNA and representative

Table 3. Effects of inoculations of wheat ears at GS 65 with *Fusarium* species on FHB severity, 1000-kernel mass of wheat, the frequency of *Fusarium* infected kernels, and biomass of *Fusarium* species in kernels

Inoculum[1]	Disease severity (1 – 9)[2]			Thousand kernel mass [g]			Frequency of infected kernels [%]			*Fusarium* biomass [pg DNA/mg kernel mass]		
	Greenhouse		Field	Greenhouse		Field	Greenhouse		Field	Greenhouse		Field
	2005	2006	2006	2005	2006	2006	2005	2006	2006	2005	2006	2006
Non-inoculated	–	–	–	25.8 a	27.7 a	36.5 a	–	–	–	–	–	–
F. avenaceum	3.4 b[3]	3.5 c	2.0 c	21.6 ab	27.7 a	36.5 a	35.2 c	28.5 c	12.3 c	1065 c	620 c	779 bc
F. culmorum	4.5 b	5.2 b	4.3 b	18.4 bc	24.5 a	32.0 b	49.6 b	47.5 b	45.3 b	9356 b	10571 b	4324 b
F. graminearum	6.5 a	7.1 a	6.7 a	12.9 c	16.1 b	28.8 c	65.2 a	63.5 a	62.5 a	15921 a	17360 a	11818 a
F. poae	1.0 c	1.0 d	1.0 d	22.5 ab	27.7 a	36.5 a	2.0 d	7.0 d	5.0 c	46 c	59 c	360 c

Notes: [1] *F. graminearum* isolate 5.1; *F. culmorum* isolate 3.11; *F. avenaceum* isolate 1.7; *F. poae* isolate 7.8;
[2] Data are the mean of three disease severity ratings (1 to 9) 7, 14 and 21 days after inoculation;
[3] Means with the same letters within columns are not significantly different (Duncan test, $p \leq 0.05$).

results. In other DNA extraction methods the sample sizes were too small or several analyses needed to be carried out to obtain reliable results. The CTAB DNA extraction method provides an efficient and versatile tool for high-throughput applications and allows the collection of a high number of data in a short time.

The PCR results confirmed the specificity of the primers reported by Waalwijk et al. [13] and Parry and Nicholson [12] for selective amplification of individual *Fusarium* species in a complex of non-target DNA either in conventional PCR or SYBR®Green real-time PCR. The SYBR® Green real-time PCR proved to be highly specific for the detection of individual species in a mixture including fungal and plant DNA. DNA from plant material and other fungi, even from other FHB pathogens, did not affect the assays. This method may be applied in surveys with a large number samples for the identification and quantification of *Fusarium* species in a complex matrix. The dynamic range for detection of *Fusarium* species varied from 9000 to 0.9 pg. For *F. culmorum* also an amount of 0.09 pg DNA gave reliable results in most experiments. The genome sequence of *F. graminearum* [30] predicts a genome size of 36 Mb (equal to 0.04 pg). Therefore, the detection limit of the assays should be less than 5 copies of genome equivalents. The validation assays indicated that amplifications using SYBR® Green real-time PCR were highly reproducible within replicates of the same sample DNA preparations as well as between different sample preparations.

In most cases, *Fusarium* identity based on fungal morphology was confirmed by using species-specific primers for PCR. In a few cases contradictory results were obtained by conventional PCR assays as compared to morphological identification of *F. poae* isolates. Further investigations showed that these isolates had been miss-identified as *F. poae* based on morphological identification and were later identified as *F. langsethiae* and *F. sporotrichioides*. The results demonstrated that molecular techniques using species-specific primers are superior tools to identify *Fusarium* species.

Quantitative PCR was superior to the microbiological assay notably in quantifying low disease levels. This applies especially to less aggressive *Fusarium* species the detection and quantification of which was challenging in conventional assays.

Comparison of the frequency of infected kernels and the concentration of *Fusarium* DNA of kernels proved consistent differences in the aggressiveness of *Fusarium* species/isolates and their development in wheat kernels irrespective of the environmental conditions. For example, under greenhouse conditions, the frequency of kernels infected by *F. graminearum* was 1.27, 1.93 and 13.7 times higher than for *F. culmorum*, *F. avenaceum* and *F. poae*, whereas the amount of fungal DNA in kernels was 1.67, 19.74 and 314 times higher, respectively. For the most aggressive species/isolates, the microbiological assessment of infected kernels and the quantitative PCR estimating fungal biomass gave similar results. For the less aggressive species, however, the intensity of kernel colonization was lower than expected from the frequency of infected kernels. These observations suggest that the frequency of infected kernels tends to overestimate the importance of less aggressive *Fusarium* species in wheat samples. *Fusarium avenaceum* is moderately pathogenic and a weak colonizer of kernels, while *F. poae* is poor in pathogenicity and aggressiveness, suggesting different mechanisms of pathogenicity and aggressiveness in the complex of *Fusarium* species causing FHB disease [31,32].

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