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A multiplex real-time PCR method using hybridization probes for the detection and the quantification of *Fusarium proliferatum*, *F. subglutinans*, *F. temperatum*, and *F. verticillioides*

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ABSTRACT

Maize contamination with *Fusarium* species is one of the major sources of mycotoxins in food and feed derivates. In the present study, a LightCycler® real-time PCR method using hybridization probes was developed for the specific identification, detection, and quantification of *Fusarium proliferatum*, *Fusarium subglutinans*, *Fusarium temperatum*, and *Fusarium verticillioides*, four mycotoxin-producing pathogens of maize. Primers and hybridization probes were designed to target the translation elongation factor 1α (*EF-1α*) gene of *F. subglutinans* and *F. temperatum* or the calmodulin (*Cal*) gene of *F. proliferatum* and *F. verticillioides*. The specificity of the real-time PCR assays was confirmed for the four *Fusarium* species, giving no amplification with DNA from other fungal species commonly recovered from maize. The assays were found to be sensitive, detecting down to 5 pg and 50 pg of *Fusarium* DNA in simplex and multiplex conditions respectively, and were able to quantify pg-amounts of *Fusarium* DNA in artificially *Fusarium*-contaminated maize samples. The real-time PCR method developed provides a useful tool for routine identification, detection, and quantification of toxigenic *Fusarium* species in maize.

Introduction

The species *Fusarium proliferatum* (Matsushima) Nirenberg, *Fusarium subglutinans* (Wollenw. & Reinking) P.E. Nelson, Toussoun & Marasas, *Fusarium temperatum* Scauflaire & Munaut and *Fusarium verticillioides* (Saccardo) Nirenberg are pathogens of maize, causing ear rot and stalk rot in tropical and temperate regions (Bottalico 1998; Leslie & Summerell 2006; Scauflaire *et al.* 2011b). In addition to crop losses and reduction of seed quality, these *Fusarium* species produce mycotoxins that accumulate in plant tissues and were proven to pose serious problems for both human and animal health

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(Logrieco et al. 2002; Desjardins 2006). Each species present a specific toxin profile, including fumonisins, fusaric acid, moniliformin, beauvericin, fusaproliferin, fusarins and enniatins (Leslie & Summerell 2006; Scauflaire et al. 2012). These four species belong to the *Gibberella fujikuroi* species complex (GFSC) which was divided into three phylogeographic clades: the African clade, which includes *F. verticillioides*; the American clade, which includes *F. subglutinans* and *F. temperatum*; and the Asian clade, which includes *F. proliferatum* (O'Donnell et al. 1998).

Due to morphological similarities of species in the GFSC, identification is usually based on various approaches combining morphological species recognition (MSR), biological species recognition (BSR) with diagnostic sexual crosses, and phylogenetic species recognition (PSR) using DNA sequence polymorphisms (Taylor et al. 2000; Kvas et al. 2009). Nevertheless, most protocols used for MSR and BSR are time-consuming, labour-intensive and require considerable expertise in *Fusarium* taxonomy and physiology, because of morphological similarities and interspecific sexual crosses that may occur under laboratory conditions (Leslie & Summerell 2006). As the rapid identification of these *Fusarium* species is critical to evaluate quality of feed samples or to assess their occurrence in fields during the growing season, there is a need to develop a complementary tool using PCR techniques that allow for rapid, specific, sensitive, and reliable diagnosis of *Fusarium* species. Among the different PCR techniques (Munaut et al. 2011), the real-time PCR using hybridization probes shows high specificity and sensitivity and combines the fast *in vitro* amplification of DNA with immediate fluorescence detection of the amplicon, allowing DNA quantification (Caplin et al. 1999; Poitras & Houde 2002). In this method, the acceptor probe is labelled with a fluorescent dye at the 5′ end and the donor probe with a fluorescein at the 3′ end. When donor and acceptor probes hybridize to adjacent regions on the target DNA in close proximity, Fluorescence Resonance Energy Transfer (FRET) interaction can occur. The fluorescent donor molecule is excited by an external light source which subsequently transfers its energy to the acceptor fluorophore. The excited acceptor emits light of a different wavelength which can be detected and measured by the LightCycler® instrument.

To achieve a rapid specific and sensitive method for the detection and the quantification of *F. proliferatum*, *F. subglutinans*, *F. temperatum*, and *F. verticillioides* in maize samples, the following steps of this work were (i) to develop species specific primers and hybridization probes designed from the translation elongation factor 1α (*EF-1α*) or the calmodulin (*Cal*) gene sequences, (ii) to evaluate the specificity and the sensitivity of the real-time PCR assays in simplex and multiplex conditions, and (iii) to validate the method by detection and quantification of the four *Fusarium* species in artificially *Fusarium*-contaminated maize samples.

Materials and methods

Fungal strains

In order to develop the real-time PCR assays, 29 strains of 13 *Fusarium* species and eight strains of eight other fungal genera commonly recovered in maize fields were used in this study (Table 1). All *Fusarium* strains were taxonomically well identified by morphological and phylogenetic characterization performed in previous studies (Britz et al. 1999; Zeller et al. 2003; Scauflaire et al. 2011a, b). The identification of each other fungal species was checked by morphological characteristics and by comparing the internal transcribed spacer (ITS) sequences of ribosomal DNA with data from GenBank.

Maize samples

Artificially *Fusarium*-contaminated maize samples were analyzed for the detection of *Fusarium proliferatum*, *Fusarium subglutinans*, *Fusarium temperatum*, and *Fusarium verticillioides* in the validation assays. The artificial contamination of stalks was performed as described by Danielsen et al. (1998) by insertion of a *Fusarium*-inoculated toothpick in the second internode of a 7-week-old maize plant. *Fusarium proliferatum* MUCL 53606, *F. subglutinans* MUCL 52467, *F. temperatum* MUCL 52463, and *F. verticillioides* MUCL 53471 were individually cultured in 10-mL tubes containing autoclaved toothpicks and 5 mL malt extract 2 % broth medium (20 g of malt extract L^{-1}, Duchefa, Haarlem, The Netherlands), and were incubated for 2 weeks at 25 °C in the dark. In addition, *Fusarium*-coinoculated toothpicks were prepared with all possible combinations of the two, three or four *Fusarium* strains. The test was duplicated and included control plants with insertion of a sterile toothpick. After 11 d, maize plants were collected and *Fusarium*-contaminated stalks were stored at −30 °C.

For kernel contamination, the four *Fusarium* strains as well as all their combinations were cultured in duplicate on 40 g of doubly autoclaved maize kernels that were adjusted to approximately 50 % moisture in 100 mL plastic flasks and inoculated with one plug of 25 mm^2 of 7-day-old mycelium grown on potato dextrose agar (PDA; Sharlau, Spain). Cultures were incubated at 25 °C for 3 weeks in the dark. Harvested culture material was lyophilized and stored at −30 °C until use. Control was similarly treated, except that it was not inoculated.

DNA extraction

To produce biomass for DNA extraction, fungal strains were grown in the dark at 25 °C for 5 d in malt extract 2 % broth medium (20 g of malt extract L^{-1}, Duchefa, Haarlem, The Netherlands) on a rotary shaker (100 rpm). Mycelium was harvested by centrifugation and the pellets were lyophilized and stored at −20 °C. To obtain genomic DNA from *Fusarium*-contaminated maize samples, 100 mg of infected tissue were sampled and were placed in a sterile tube. Both lyophilized mycelia and maize tissues were disrupted in a sterile tube with silica beads (Biospec Products, Bartlesville, USA) in the MagNA Lyser cell disrupter (Roche Diagnostics, Mannheim, Germany). Fungal DNA was extracted and purified using the Invisorb Spin Plant MiniKit (Invitek, Berlin, Germany) according to the manufacturer's recommendations. Purified DNA was quantified by the NanoDrop ND-1000 Spectrophotometer (Saveen Werner, Malmö, Sweden) and stored at −80 °C.

Table 1 – Specificity of real-time PCR assay for the detection of *F. proliferatum*, *F. subglutinans*, *F. temperatum*, and *F. verticillioides* against various fungal species.

Fungal species[a]	Strain number[b]	FPROLI/FSUB/FTEMP/ FVERTI PCR assays[c]	Reference
F. proliferatum	MUCL 53606	+/–/–/–	Scauflaire *et al.* 2011b
	MUCL 53607	+/–/–/–	Scauflaire *et al.* 2011b
	IL096	+/–/–/–	Scauflaire *et al.* 2011b
	IIH048	+/–/–/–	Scauflaire *et al.* 2011b
	IIL033	+/–/–/–	Scauflaire *et al.* 2011b
F. subglutinans	MUCL 52466	–/+/–/–	Scauflaire *et al.* 2011b
	MUCL 52467	–/+/–/–	Scauflaire *et al.* 2011b
	MUCL 52468	–/+/–/–	Scauflaire *et al.* 2011a
	IIIC032	–/+/–/–	Scauflaire *et al.* 2011b
	IVB055	–/+/–/–	Scauflaire *et al.* 2011b
F. temperatum	MUCL 52438	–/–/+/–	Scauflaire *et al.* 2011a
	MUCL 52445	–/–/+/–	Scauflaire *et al.* 2011a
	MUCL 52447	–/–/+/–	Scauflaire *et al.* 2011a
	MUCL 52454	–/–/+/–	Scauflaire *et al.* 2011a
	MUCL 52463	–/–/+/–	Scauflaire *et al.* 2011a
F. verticillioides	MUCL 53471	–/–/–/+	Scauflaire *et al.* 2011b
	MUCL 53472	–/–/–/+	Scauflaire *et al.* 2011b
	IM102	–/–/–/+	Scauflaire *et al.* 2011b
	IIR078	–/–/–/+	Scauflaire *et al.* 2011b
	IVB133	–/–/–/+	Scauflaire *et al.* 2011b
F. avenaceum	MUCL 53608	–/–/–/–	Scauflaire *et al.* 2011b
F. circinatum	MUCL 47029	–/–/–/–	Britz *et al.* 1999
F. crookwellense	MUCL 53462	–/–/–/–	Scauflaire *et al.* 2011b
F. culmorum	MUCL 53470	–/–/–/–	Scauflaire *et al.* 2011b
F. equiseti	MUCL 53609	–/–/–/–	Scauflaire *et al.* 2011b
F. graminearum	MUCL 53610	–/–/–/–	Scauflaire *et al.* 2011b
F. konzum	MUCL 47031	–/–/–/–	Zeller *et al.* 2003
F. oxysporum	MUCL 53611	–/–/–/–	Scauflaire *et al.* 2011b
F. poae	MUCL 53185	–/–/–/–	Scauflaire *et al.* 2011b
Acremonium strictum	MUCL 53612	–/–/–/–	This study
Alternaria alternata	MUCL 53613	–/–/–/–	This study
Cladosporium cladosporioides	MUCL 53614	–/–/–/–	This study
Epicoccum nigrum	MUCL 53615	–/–/–/–	This study
Microdochium bolleyi	MUCL 53616	–/–/–/–	This study
Didymella exitialis	MUCL 53617	–/–/–/–	This study
Torrubiella confragosa	MUCL 53618	–/–/–/–	This study
Trichoderma koningii	MUCL 53619	–/–/–/–	This study

a Except for *F. circinatum* (*Pinus* sp./South Africa) and *F. konzum* (*Andropogon* sp./USA), all species used in this study were collected from maize in Belgium.
b MUCL = Mycothèque de l'Université catholique de Louvain, Louvain-la-Neuve, Belgium.
c + and – indicate the presence and absence of the expected DNA fragment amplified and detected by PCR assays FPROLI, FSUB, FTEMP, and FVERTI, respectively.

PCR primers and hybridization probes

To avoid unspecific cross-hybridizations of the probes in multiplex conditions, the primer set of each species was designed from a different region of DNA. Primers and probes specific to *Fusarium proliferatum* and *Fusarium verticillioides* were derived from two different regions of the *Cal* gene sequence, while those specific to *Fusarium subglutinans* and *Fusarium temperatum* were designed on two different regions of the *EF-1α* gene sequence (Table 2). Both gene sequences have been successfully used to distinguish taxonomic groups within the genus *Fusarium* (Carbone & Kohn 1999; O'Donnell *et al.* 2000). *Cal* and *EF-1α* sequences from the four *Fusarium* species studied were obtained from GenBank and were aligned with Clustal W2 (Larkin *et al.* 2007). Potential primer and probe sequences were selected using LightCycler® Probe Design Software 2.0 (Roche Diagnostics, Mannheim, Germany) and BLAST analyses (Altschul *et al.* 1997) were performed to check the primers specificity against other closely related genome sequences. Primers and probes were obtained from Sigma–Aldrich N.V./S.A., Bornem, Belgium. For each pair of probes, the first one is labeled at the 5′-end with a LightCycler® Red fluorophore (610, 640, 670 or 705 nm) and the second one is labeled at the 3′-end with fluorescein, which is excited by the light source of the LightCycler® instrument.

Simplex real-time PCR assay

The LightCycler® 2.0 instrument (Roche Diagnostics, Mannheim, Germany) was used for amplification and

Table 2 – Real-time PCR primers and hybridization probes used in this study.

Fusarium species	GenBank accession #[a]	Primers/ probes[b]	Sequence (5′–3′)[c]	Targeted gene	Tm[d] (°C)
F. proliferatum strain CE1	EU430620	Fproli-F	TATTTCGCCGCCTTGTCTC	*Cal*	60.2
(Visentin *et al.* 2009)		Fproli-R	GTTTAGCTCATGTTTTCGCTTCA	*Cal*	58.9
		Fproli-P1	CTCCCCTTTCCGCCAAGTTTCT-Fluorescein	*Cal*	65.0
		Fproli-P2	LC Red 640-GTTGGCCCCGTGCGAAACCC	*Cal*	67.5
F. subglutinans strain	HM057336	Fsub-F	GCTAAGCAGTCACTAACCATTCAA	*EF-1α*	59.6
NRRL 22016		Fsub-R	GTTAGTACGAATAAAGGGAGAACAAT	*EF-1α*	59.6
(O'Donnell *et al.* 2000)		Fsub-P1	CTCTGGAAGTTCGAGACTCCTCGCTA-Fluorescein	*EF-1α*	65.1
		Fsub-P2	LC Red 670-ATGTCACCGTCATTGGTATGTTGTCGCTC	*EF-1α*	67.9
F. temperatum strain	HM067687	Ftemp-F	AAGACCTGGCGGGC	*EF-1α*	60.5
MUCL 52450		Ftemp-R	TCAGAAGGTTGTGGCAATGG	*EF-1α*	60.0
(Scauflaire *et al.* 2011a)		Ftemp-P1	ACAAGCGAACCATCGAGAAGTTCGA-Fluorescein	*EF-1α*	65.6
		Ftemp-P2	LC Red 705-AGGTTAGTTACTTTCCCTTCGATCGCGC	*EF-1α*	67.6
F. verticillioides strain	AF158315	Fverti-F	CTTCCTGCGATGTTTCTCC[e]	*Cal*	58.4
NRRL 22172		Fverti-R	CGACCGTCCTATAATCGTC	*Cal*	59.2
(O'Donnell *et al.* 2000)		Fverti-P1	CCTCGCTCTAGGCCAGATTACCAC-Fluorescein	*Cal*	65.4
		Fverti-P2	LC Red 610-AGGAGCTCGGTACCGTTATGCG	*Cal*	66.9

a GenBank accession numbers (NCBI database) for the *Fusarium* DNA used to develop primers and probes.
b F for forward primer, R for reverse primer, and P for hybridization probe.
c LC Red [wavelengthxxx]: LightCycler Red-[wavelengthxxx]-*N*-hydroxy-succinimide ester.
d Tm for melting temperature (theorical hybridization temperature).
e This primer corresponds to the primer PRO1 from Mulè *et al.* (2004).

quantification of *Fusarium* DNA. The real-time PCR was performed in glass capillaries (LightCycler® Capillaries, Roche Diagnostics) with a LightCycler® FastStart DNA Master Plus HybProbes kit (Roche Diagnostics) as described by the manufacturer's instructions. The PCR reaction mixture contained 4 μL of a 5× Master Mix (containing dNTP's, FastStart Taq DNA polymerase, reaction buffer, and $MgCl_2$), 0.5 μM of each primer, 0.2 μM of each probe, and 5 μL of template DNA in a final volume of 20 μL. PCR was performed under the following conditions: pre-incubation step for 10 min at 95 °C, 40 cycles for 10 s at 95 °C, annealing for 10 s at 63–65 °C depending on Tm of primers, enzymatic chain extension for 8 s at 72 °C and a final extension step at 72 °C. Fluorescence was monitored between the annealing and elongation steps. For each PCR assay an internal negative control was included, which was run without the addition of template DNA.

Specificity, sensitivity, and DNA quantification

The specificity of the PCR assays was tested in duplicate against genomic DNA of 21 fungal species: the four *Fusarium* species for which the assay was developed (*Fusarium proliferatum*, *Fusarium subglutinans*, *Fusarium temperatum*, and *Fusarium verticillioides*), seven different *Fusarium* species commonly recovered from maize plants (*Fusarium avenaceum*, *Fusarium crookwellense*, *F. culmorum*, *Fusarium equiseti*, *Fusarium graminearum*, *Fusarium oxysporum*, and *Fusarium poae*) and eight different other fungal species that are contaminant in maize fields (*Acremonium strictum*, *Alternaria alternata*, *Cladosporium cladosporioides*, *Didymella exitialis*, *Epicoccum nigrum*, *Microdochium bolleyi*, *Torrubiella confragosa*, and *Trichoderma koningii*). In addition, two *Fusarium* species (*Fusarium circinatum* and *Fusarium konzum*) phylogenetically closely related to *F. subglutinans* and *F. temperatum* were added for testing specificity, although they were not recovered from maize. DNA extracted from healthy maize was used as an external negative control to exclude any cross-reactivities of the primers and the hybridization probes with maize DNA. The sensitivity and linear range of the real-time PCR assays were determined in triplicate using tenfold serial dilution of genomic DNA from *F. proliferatum* MUCL 53607, *F. subglutinans* MUCL 52468, *F. temperatum* MUCL 52463, and *F. verticillioides* MUCL 53472. Crossing point (Cp) values were recorded by online monitoring when the logarithmic linear phase was distinguished from the threshold. Cp values of the tenfold serial dilution of genomic DNA were used to generate standard curves. The concentration of *Fusarium* DNA in a maize sample was calculated by comparing the Cp value obtained for the sample within the corresponding standard curve. In order to minimize false positive results due to unspecific amplification during the late PCR cycles, a PCR reaction was considered positive only if the Cp value was <35.

Multiplex real-time PCR assay

The multiplex real-time PCR assay consisted of a multicolour reaction in which DNA template from four different *Fusarium* species were detected in a single capillary. LightCycler® Multiplex DNA Master HybProbe kit (Roche Diagnostics) was used to perform the multiplex PCR assays. The reaction mix consisted of 4 μL of a 5× Master Mix, 0.5 μM of each primer, 0.2 μM of each probe, and 2 μL of DNA template in a final volume of 20 μL. After a pre-incubation step for 10 min at 95 °C, amplification was performed during 40 cycles of 10 s at 95 °C, annealing of 10 s at 63 °C, and extension of 8 s at 72 °C.

The sensitivity of the multiplex PCR assay was also determined in triplicate by detecting each of the four *Fusarium* species (*Fusarium proliferatum*, *Fusarium subglutinans*, *Fusarium temperatum* or *Fusarium verticillioides*) DNA in tenfold serial dilution of mixed genomic DNA from the four species.

Results

Selection and specificity of primers and hybridization probes

The alignments of the *Cal* and *EF-1α* gene sequences of the four target species *Fusarium proliferatum*, *Fusarium subglutinans*, *Fusarium temperatum*, and *Fusarium verticillioides* displayed high nucleotide divergences which were used for the selection of primers and hybridization probes (data not shown). Thereafter, primers were checked by comparing sequence homology with DNA sequences available from GenBank to confirm their specificity to their corresponding *Fusarium* species. Primers and hybridization probes were designed to work simultaneously under the same PCR conditions (Table 2).

For *F. proliferatum*, the 221-bp *Cal* gene fragment flanked by primers Fproli-F and Fproli-R was detected by probes Fproli-P1 and Fproli-P2 (FPROLI assay). For *F. subglutinans*, primers Fsub-F and Fsub-R allowed amplification of a 213-bp *EF-1α* gene fragment detected by probes Fsub-P1 and Fsub-P2 (FSUB assay). For *F. temperatum*, Ftemp-F and Ftemp-R were used as primers to amplify a 296-bp *EF-1α* gene fragment detected by probes Ftemp-P1 and Ftemp-P2 (FTEMP assay). Finally, primers Fverti-F and Fverti-R amplified a 302-bp *Cal* gene fragment of *F. verticillioides*, detected by probes Fverti-P1 and Fverti-P2 (FVERTI assay). No intra-species variation of the primer and probe regions was observed between the five strains of each of the four species for which the assay was developed and the strain from GenBank.

The species specificity of the real-time PCR assays was tested against genomic DNA of target and non-target *Fusarium* strains, as well as from other fungal species. The primers and hybridization probes sets of the four species studied were highly specific as shown in Table 1. No signals or only weak signals (Cp values >35) due to unspecific amplification were detected with the nontarget species. No cross-reactivity with DNA extracted from maize grown under sterile conditions was observed.

Sensitivity and reproducibility in simplex conditions

Detection limit and standard deviation of the PCR assays were measured using serial dilution of *Fusarium* DNA in three independent experiments. The real-time PCR assays in simplex conditions detected down to 11 pg, 8 pg, 5 pg, and 5 pg of *Fusarium proliferatum* DNA, *Fusarium subglutinans* DNA, *Fusarium temperatum* DNA, and *Fusarium verticillioides* DNA, respectively, with linear ranges of five orders of magnitude (Fig 1). The correlations between the Cp values and known DNA quantities were high for the four assays, i.e. r^2 (FPROLI) = 0.9992, r^2 (FSUB) = 0.9965, r^2 (FTEMP) = 0.9971, and r^2 (FVERTI) = 0.996. The coefficient of variation did not exceed 3.4 % and the linear regression slopes ranged between 3.2913 and 3.4077.

Sensitivity and reproducibility in quadruplex conditions

The detection limit for the multiplex real-time PCR assay was assessed under an optimized annealing temperature of 63 °C with a mixture of DNA extracted from pure cultures of *Fusarium proliferatum*, *Fusarium subglutinans*, *Fusarium temperatum*, and *Fusarium verticillioides*. Serial dilutions were made of DNA from the four fungal species. For each *Fusarium* species tested, the real-time PCR assay in quadruplex conditions

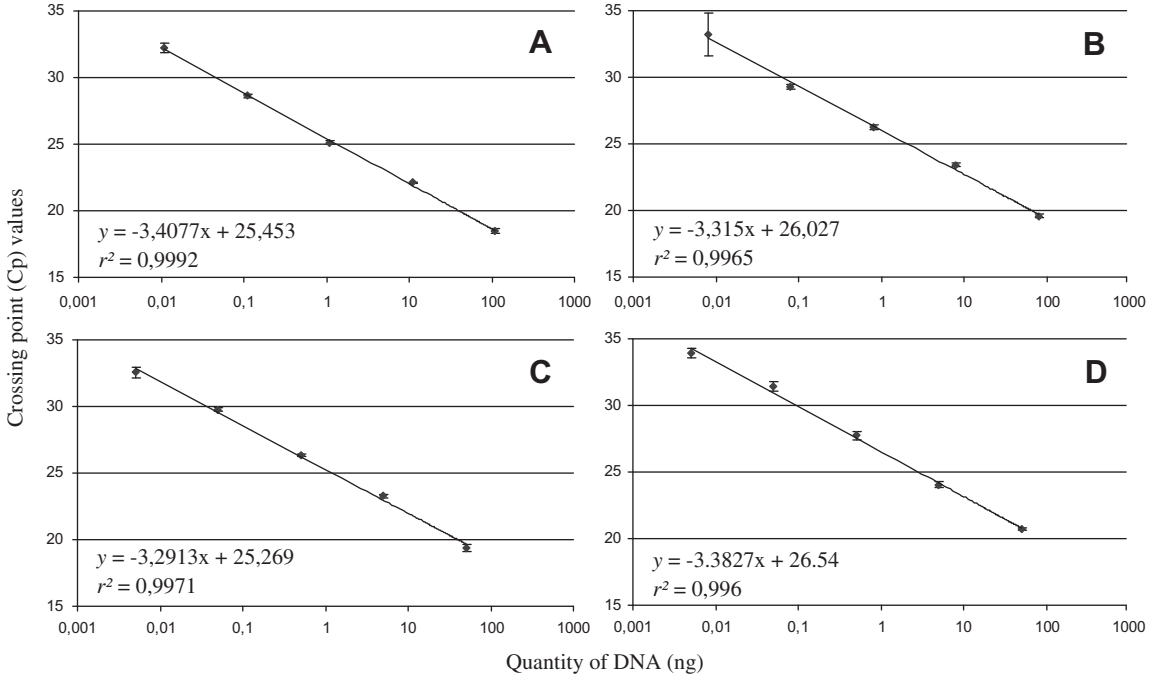

Fig 1 – Standard curves generated for *F. proliferatum* (A), *F. subglutinans* (B), *F. temperatum* (C), and *F. verticillioides* (D) by simplex real-time PCR. The Cp values were plotted against the DNA concentrations expressed on a logarithmic scale. The error bars indicate the standard deviations of Cp among three replicates.

detected down to 50 pg of DNA with linear ranges of three orders of magnitude (Fig 2). High correlations between the Cp values and the DNA quantities were observed for FPROLI ($r^2 = 0.9986$), FSUB ($r^2 = 0.9899$), and FVERTI ($r^2 = 0.9994$), while the correlation for FTEMP ($r^2 = 0.9761$) was acceptable. The coefficient of variation was max. 4.7 % and the linear regression slopes ranged between 2.1533 and 3.1717.

Validation with maize samples

Artificially *Fusarium*-contaminated stalks and kernels were analyzed to evaluate the ability of the quadruplex real-time PCR assay to detect and quantify the four *Fusarium* species in field samples. All expected *Fusarium* species as well as their combinations were detected in the maize samples (Table 3). The ranges of fungal DNA recovered in infected stalks were 36–793, 3–1491, 18–4623, and 62–717 pg mg^{-1} of maize tissue for *Fusarium proliferatum*, *Fusarium subglutinans*, *Fusarium temperatum*, and *Fusarium verticillioides*, respectively. Comparatively, the amounts of fungal DNA detected in infected kernels were lower than those detected in stalks and the ranges were 4–55, 28–380, <1–7, and 7–208 pg mg^{-1} of maize tissue for *F. proliferatum*, *F. subglutinans*, *F. temperatum*, and *F. verticillioides*, respectively. The coefficient of variation did not exceed 3 % when the Cp values of the DNA samples analyzed in duplicate were compared (data not shown). No amplification occurred when healthy maize DNA was analyzed by the assays. An unexpected amplification of *F. temperatum* occurred when analyzing the stalk sample artificially contaminated with the *F. proliferatum*/*F. subglutinans* combination, suggesting a *F. temperatum* contamination of the stalk sample or a limitation of multiplex assay. The hypothesis of sample contamination during the test has been retained, as their was no amplification of *F. temperatum* when running a PCR assay with the DNA from a tissue mixture of two samples that had been inoculated with a single species of *F. proliferatum* and *F. subglutinans*.

Discussion

The evaluation of *Fusarium* spp. contaminations in maize samples is essential for studies on population dynamics, epidemiology of disease, and mycotoxin production estimations. However, visual assessment of infection in the field and plating methods are time-consuming and require substantial expertise for species identification. Real-time PCR offers an alternative tool for efficient estimation of DNA quantities of individual species. In this study, a rapid specific and sensitive real-time PCR assay was developed to detect and quantify four *Fusarium* species, *Fusarium proliferatum*, *Fusarium subglutinans*, *Fusarium temperatum*, and *Fusarium verticillioides*, that are mycotoxin-producing pathogens of maize.

The primers and the hybridization probes used in the assays were designed to amplify distinct fragments of the *EF-1α* and *Cal* genes. Those genes are well studied and a large number of sequences are available in the GenBank database. Furthermore those genes allow the discrimination of most *Fusarium* species (Kristensen *et al.* 2005; Kvas *et al.* 2009). Compared to other PCR chemistries, the LightCycler® PCR assays using hybridization probes featured enhanced species specificity due to the use of two independent hybridization probes per *Fusarium* sp. studied. In the specificity tests, no cross-amplifications were observed with DNA from maize, from

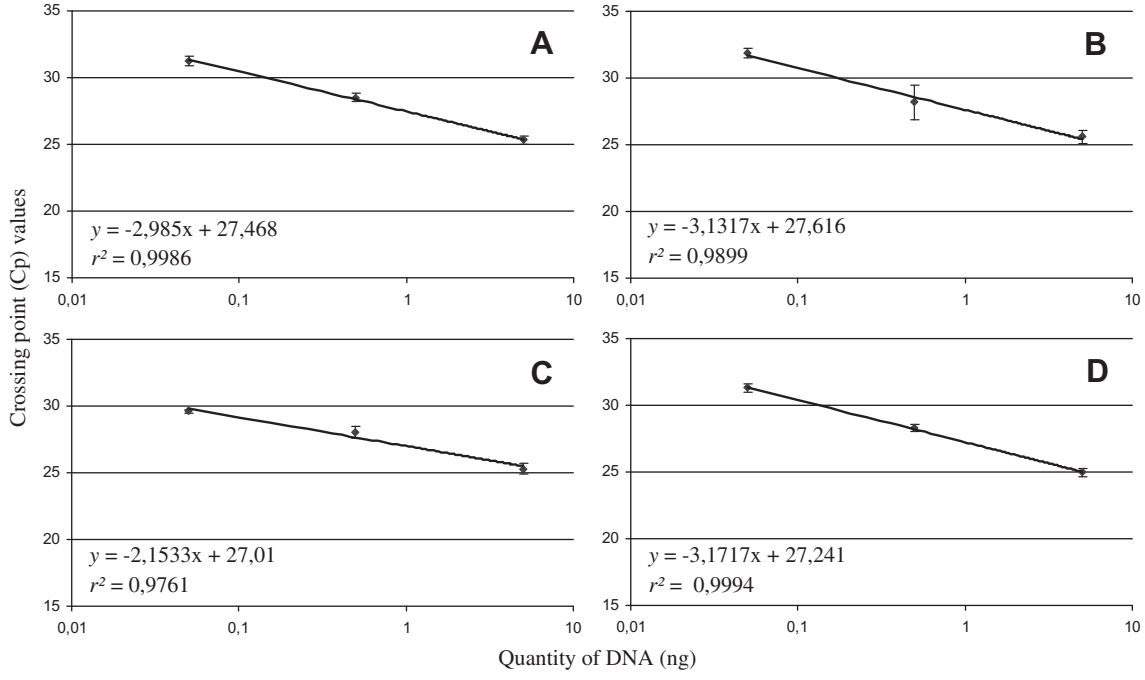

Fig 2 – Standard curves generated for *F. proliferatum* (A), *F. subglutinans* (B), *F. temperatum* (C), and *F. verticillioides* (D) by multiplex real-time PCR. The Cp values were plotted against the DNA concentrations expressed on a logarithmic scale. The error bars indicate the standard deviations of Cp among three replicates.

Table 3 – Detection and quantification of *Fusarium* species in artificially *Fusarium*-contaminated maize samples by multiplex real-time PCR assay.

Fusarium inoculation[a]				Multiplex assay (stalk/kernel tissue)[b]			
proli	sub	temp	verti	FPROLI	FSUB	FTEMP	FVERTI
+	–	–	–	130/31	ND	ND	ND
–	+	–	–	ND	149/380	ND	ND
–	–	+	–	ND	ND	4623/1	ND
–	–	–	+	ND	ND	ND	618/18
+	+	–	–	558/16	228/297	87/ND	ND
+	–	+	–	38/5	ND	129/<1	ND
+	–	–	+	793/55	ND	ND	717/208
–	+	+	–	ND	74/297	176/<1	ND
–	+	–	+	ND	1491/140	ND	251/66
–	–	+	+	ND	ND	516/<1	168/28
–	+	+	+	ND	3/56	28/1	62/7
+	–	+	+	36/17	ND	18/2	67/33
+	+	–	+	98/5	100/28	ND	208/31
+	+	+	–	37/4	63/126	1366/2	ND
+	+	+	+	49/26	100/132	530/7	560/52
–	–	–	–	ND	ND	ND	ND

a proli = *F. proliferatum*; sub = *F. subglutinans*; temp = *F. temperatum*; verti = *F. verticillioides*. + and – indicate the presence and absence of the *Fusarium* species used for stalk or kernel inoculation.
b pg of DNA per mg of maize infected tissue, according to the Cp values (mean of two replicates); ND = not detected.

genetically closely related *Fusarium* species, or from a wide range of other fungal species commonly recovered in the field.

The detection limit of the assays in simplex conditions was 5–10 pg of DNA, which was similar to those reported in other studies using single copy nuclear genes for real-time PCR method (Bluhm *et al.* 2004; Reischer *et al.* 2004; Fredlund *et al.* 2008; Godet & Munaut 2010). The detection limit of the assays in quadruplex conditions was 50 pg of DNA. This difference in sensitivity of one order of magnitude between simplex and multiplex conditions has already been observed (Bezuidenhout *et al.* 2006) and was probably due to primers and probes competition effects. The sensitivity was also tested with dilution series of *Fusarium* and maize DNA's to assess the ability of the real-time PCR to quantify fungal biomass in plant tissue. The presence of as much as 135 ng of maize DNA in the PCR capillaries did not interfere with the quantification of small amounts of target DNA, which indicated that the concentration of dNTPs was sufficient to overcome competition effects (Suanthie *et al.* 2009).

The standard curves of the simplex real-time PCR showed slopes of −3.3 to −3.4 with r^2 values >0.99. Although no guidelines were established for standard curves obtained from real-time PCR assays on fungal materials, these values are in agreement with the minimum performance requirements for analytical methods of Genetically Modified Organism (GMO) testing in food and animal feed (European Network of GMO Laboratories 2008). Indeed, slopes of the linear regression of real-time PCR methods should range between −3.1 and −3.6, corresponding to a PCR efficiency of 80–100 %, and r^2 values should be ≥0.98. In quadruplex conditions, these minimum performance requirements are only obtained for the detection of *F. subglutinans* and *F. verticillioides*. The slopes of the standard curves of *F. proliferatum* and *F. temperatum*, −3 and −2.2 respectively, indicated a diminution of PCR efficiency when multiplexed, although acceptable coefficients of variation and r^2 values.

This is the first report of a quadruplex real-time PCR method including the detection and the quantification of *F. temperatum*. The method also allows the distinction between *F. temperatum* and *F. subglutinans* in maize samples, two morphologically similar and phylogenetically closely related species that need to be distinguished due to their differences in pathogenicity and mycotoxin productions (Scauflaire *et al.* 2012). Furthermore, the present study demonstrates that the LightCycler® real-time PCR technology using hybridization probes is an efficient tool to rapidly, specifically and sensitively detect contaminations by *F. proliferatum*, *F. subglutinans*, *F. temperatum* or *F. verticillioides* in stalks of maize but also in kernels.

In conclusion, the method represents a useful tool to evaluate quality of maize materials at different critical points of the feed chain. The fungal DNA quantification developed in this work could also be used in epidemiological studies, such as estimation of the inoculum potential, or screening of resistant maize materials. The multiplex real-time PCR assays might be used as a valid diagnosis tool for routine identifications of the four *Fusarium* species in cereal samples, without any mycological expertise. Further research will focus on *Fusarium* sp. detection from field samples and on the correlation between the quantities of DNA detected in plant tissues and mycotoxin amounts in the contaminated samples.

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