Quantification of *Fusarium graminearum* and *Fusarium culmorum* by real-time PCR system and zearalenone assessment in maize

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A B S T R A C T

Zearalenone (ZEA) is a mycotoxin produced by some species of *Fusarium*, especially by *Fusarium graminearum* and *F. culmorum*. ZEA induces hyperoestrogenic responses in mammals and can result in reproductive disorders in farm animals. In the present study, a real-time PCR (qPCR) assay has been successfully developed for the detection and quantification of *Fusarium graminearum* based on primers targeting the gene *PKS13* involved in ZEA biosynthesis. A standard curve was developed by plotting the logarithm of known concentrations of *F. graminearum* DNA against the cycle threshold (Ct) value. The developed real time PCR system was also used to analyze the occurrence of zearalenone producing *F. graminearum* strains on maize. In this context, DNA extractions were performed from thirty-two maize samples, and subjected to real time PCR. Maize samples also were analyzed for zearalenone content by HPLC. *F. graminearum* DNA content (pg DNA/ mg of maize) was then plotted against ZEA content (ppb) in maize samples. The regression curve showed a positive and good correlation ($R^2 = 0.760$) allowing for the estimation of the potential risk from ZEA contamination. Consequently, this work offers a quick alternative to conventional methods of ZEA quantification and mycological detection and quantification of *F. graminearum* in maize.

1. Introduction

Zearalenone (ZEA) is a polyketide mycotoxin produced by some species of *Gibberella*/*Fusarium*, especially by *Fusarium graminearum (Gibberella zeae)* and *F. culmorum* (Kim et al., 2005). It is found in a number of cereal crops such as maize, barley, oats, wheat, and rice from the continents of Australia, Europe, and North America, as well as in New Zealand (Suzuki et al., 2007; Alldrick and Hajšelová, 2004). The occurrence of ZEA in food and feed has also been demonstrated in South America, Africa, Taiwan, China, and Russia (Suzuki et al., 2007). Consequently, the question of ZEA contamination is one which has to be addressed worldwide (Alldrick and Hajšelová, 2004).

ZEA causes alterations in the reproductive tract of laboratory and domestic animals. In addition, various estrogenic effects, such as decreased fertility, increased fetal resorptions, and changes in the weight of endocrine glands and serum hormone levels have been observed (Suzuki et al., 2007). Due to the health hazards of ZEA, the European Commission has set a maximum level of 100 μg/kg for ZEA in unprocessed cereals excluding maize (European Commission Regulation No 1126/2007). The permitted level in unprocessed maize set by the European commission was 350 μg/kg.

As with the majority of mycotoxins, ZEA is a stable compound, both during storage/milling and processing/cooking of food, and does not decompose at high temperatures. Since the different *Fusarium* species have different mycotoxin profiles, the accurate determination of the *Fusarium* species present in any foodstuffs is critical to predict the potential risk of the *Fusarium* isolate and its representative mycotoxin (s). Therefore, there is a need for developing tools which permit a rapid, sensitive and specific diagnostic of *Fusarium* species in contaminated food samples (Jurado et al., 2006). Conventional methods to assess mold presence in cereal crops are labour and time-consuming, and they are particularly complex in *Fusarium*, since the genus is diverse, presents intraspecific variability, and conflicting taxonomy (Jurado et al., 2005; Seifert and Lévesque, 2004; Edwards et al., 2002).

The polymerase chain reaction (PCR) is a rapid and specific method; its high sensitivity allows detection of target DNA molecules in a complex mixture, offering an alternative to microbiological conventional procedures in fungal diagnostic (Jurado et al., 2006; Edwards et al., 2002; Nicholson et al., 1998). One of the most important factors in the development of such molecular methods is the reliability of the primer set designed and the targeted DNA sequence of interest organism (EL Khoury and Atoui,

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2010; Atoui et al., 2007; Niessen, 2007; Dao et al., 2005; Geisen et al., 2004; Farber et al., 1997). Recent advances in DNA-based techniques such as real-time PCR (qPCR) are providing new tools for fungal detection and quantification by detecting and quantifying their DNA. qPCR can be performed using different chemistries, such as SYBR® Green I dye and TaqMan® (Casey and Dobson, 2004; EL Khoury and Atoui, 2010). Nowadays, several qPCR assays have been reported for detection of toxigenic molds using such systems (Atoui et al., 2007; Fredlund et al., 2008; Selma et al., 2008; Nicolaisen et al., 2009; Suanthie et al., 2009; Meng et al., 2010).

Recently, Meng et al. (2010) described the first report describing the development of molecular method for the detection and quantification of zearalenone-producing *Fusarium* species in foodstuff by real-time PCR assay using SYBR Green I. However, no qPCR protocol has yet been developed to estimate ZEA content in foodstuffs. In this study, we described the development of quantitative real-time PCR assay for the detection and quantification of *F. graminearum* in maize samples, and to correlate *F. graminearum* DNA with ZEA content in maize in order to have an approximately estimation of the ZEA contamination level.

2. Material and methods

2.1. Fungal strains and culture conditions

The fungal strains used in this study are described in Table 1. Strains were grown at 25 °C on potato dextrose agar (PDA) (Difco, Fisher Bioblock Scientific, Illkirch, France) during 10 days. Then spores were collected with a sterile solution of 0.1% (v/v) Tween 80 (Fisher Bioblock Scientific, Illkirch, France) and stored at – 20 °C in 25% (v/v) of glycerol (Fisher Bioblock Scientific, Illkirch, France) before use.

For DNA extractions, fungal strains were cultured in 250-ml Erlenmeyer flasks containing 100 ml of potato dextrose broth (PDB) (Difco, Fisher Bioblock Scientific, Illkirch, France). Broths were inoculated with 10^6 spores and incubated at 25 °C under static conditions for 4 days. The mycelium was then harvested by filtration through a 0.45 μm Millipore filter (Millipore Corporation, Bellerica, MA, USA), frozen in liquid nitrogen and then stored at – 80 °C before nucleic acid extraction.

Table 1
Fungal strains used in this study to test the specificity of the primer pair ZEA-F/ZEA-R.

Species	Produced mycotoxins
Fusarium graminearum NRRL 5883 (*)	Zearalenone, Deoxinivalenol
F. graminearum NRRL 28336	Zearalenone, Deoxinivalenol
F. culmorum NRRL 3288	Zearalenone, Deoxinivalenol
F. culmorum NRRL 25475	Zearalenone, Deoxinivalenol
F. proliferatum NRRL 26191	Fumonisin
F. proliferatum NRRL 6322	Fumonisin
F. moniliforme NRRL 13616	Fumonisin B1 ; B2
F. verticillioides NRRL 6442	Fumonisin B1, B2, B3, B4
F. verticillioides NRRL 34281	Fumonisin B1
F. sporotrichioides NRRL 13440	T2 toxin, HT-2 toxin
F. dlamini NRRL 13164	Fumonisin B1, fusarin
F. poae NRRL 3287	T2 toxin
F. poae CBS 317.73 (**)	T2 toxin
Aspergillus parasiticus CBS 100926	Aflatoxins B1, B2, G1, G2
A. flavus NRRL 35691	Aflatoxins B1, B2
A. ochraceus NRRL 5175	Ochratoxin A
A. westerdijkiae NRRL 3174	Ochratoxin A, Penicillic acid
A. niger CBS 120166	Ochratoxin A
A. carbonarius CBS 120168	Ochratoxin A
A. sulfureus NRRL 4077	Ochratoxin A
Penicillium verrucosum NRRL 3711	Ochratoxin A
P. nordicum (***)	Ochratoxin A
P. citrinum NRRL 1843	Citrinin
P. expansum NRRL 35694	Patulin

*: NRRL: Northern Regional Research Laboratory, Illinois, USA.
**: CBS: Centraalbureau voor Schimmel Cultures, The Netherlands.
***: Provided by Olivier Puel, INRA Toulouse.

2.2. DNA extraction from pure fungal cultures

About 200 mg of frozen mycelium was homogenized in 800 μl of lysis buffer (100 mM Tris– HCl pH 7.4 (Sigma Aldrich, Saint Quentin Fallavier, France), 20 mM EDTA (Sigma Aldrich, Saint Quentin Fallavier, France), 250 mM NaCl (Sigma Aldrich, Saint Quentin Fallavier, France), 2% w/v SDS (Sigma Aldrich, Saint Quentin Fallavier, France)) by using a Ultra- Turax (Labo moderne) and incubated at 37 °C for 30 min with 10 μl of 25 mg/ml RNase solution (Promega, Charbonnières, France), then added with 10 μl of proteinase K (20 mg/ml, Promega, Charbonnières, France) and the mixture was incubated at 65 °C for 30 min. A volume of phenol-chloroform-isoamylic alcohol (v/v/v: 25/24/1) (Sigma Aldrich, Saint Quentin Fallavier, France) was added, and the mixture was vigorously vortexed for 5 min. The aqueous phase collected after centrifugation (15,000 x g, 15 min) was extracted by an equal volume of chloroform (Sigma Aldrich, Saint Quentin Fallavier, France). Genomic DNA was precipitated at −20 °C in 2 h with two volumes of 100% ethanol (Fisher Bioblock Scientific, Illkirch, France). The DNA was pelleted by centrifuging at 15,000 x g and washed with 1 ml of 75% ethanol then dried at the room temperature. 100 μl of water was used to resuspend genomic DNA. The quality and quantity of DNA were estimated by the $OD_{260/280}$ ratio (using Philips PU 8600 Spectrophotometer) and agarose (Promega, Charbonnières, France) gel electrophoresis according to standard protocol (Sambrook et al., 1989).

2.3. DNA extraction from maize samples

About 20 g of maize grains was ground in a coffee grinder for 2 min to a fine powder and then 200 mg ground grain was weighed into a 2 mL microcentrifuge. One milliliter of sodium dodecyl sulphate (SDS) extraction buffer (200 Mm Tris–HCl, pH 7.5; 288 mM NaCl; 25 mM EDTA, pH8.0; 0.5% SDS) was added to each ground sample and vortexed for homogenization. Samples were then centrifuged at 12,000 rpm for 5 min and 750 μL of the supernatant was transferred to a new microcentrifuge tube. A 215 μL aliquot of a solution of potassium acetate (3 M potassium and 5 M acetate) was added to the supernatant. The solution was mixed and incubated on ice for 30 min, and then centrifuged at 12,000 rpm for 15 min at 4 °C. A 700 μL aliquot of the supernatant was transferred to a new microcentrifuge tube and 500 μL of cold isopropanol was added to precipitate the DNA. The resulting pellet was washed with 70% ethanol, dried under a stream of nitrogen and then dissolved in 200 μL Tris–EDTA.

2.4. Development and evaluation of specific PCR primers

A primer pair ZEA-F/ZEA-R was designed, using the Primer Express software (Applied Biosystems, Foster City, CA, USA) from a polyketide synthase gene *PKS13* (Accession number: DQ019316) involved in ZEA biosynthesis in *F. graminearum* (Gaffoor and Trail, 2006; Kim et al., 2005; Kroken et al., 2003). Primer sequences are presented in Table 2. This set of primers amplified a product of 192 bp in *F. graminearum*. Primer synthesis was performed from Eurogentec s.a., Seraing, Belgium.

Table 2
Primers used in this study.

Oligo name	Sequence
ZEA-F	5'- CTGAGAAATATCGCTACACTACCGAC-3'
ZEA-R	5'- CCCACTCAGGTTGATTTTCGTC -3'
TubF	5'- CTCGAGCGTATGAACGTCTAC-3'
TubR	5'- AAACCCTGGAGGCAGTCGC-3'

To test the specificity of the primer pair, the isolated DNA of fungal strains (Table 1) was subjected to PCR with this primer set. The PCR was performed with the *Taq* recombinant polymerase (Invitrogen, Cergy Pontoise, France). Amplification was carried out in 50 µl reaction mixture containing: 5 µl of *Taq* polymerase buffer 10 X, 1.5 µl of 50 mM $MgCl_2$, 1 µl of dNTP 10 mM of each (Promega, Charbonnières, France), 1 µM of each primer, 1.5 U of *Taq*, about 100 ng of genomic DNA, H_2O up to 50 µl. Reaction conditions were: 94 °C for 4 min, (94 °C for 45 s, 60 °C for 45 s and 72 °C for 45 s) x 35 cycles followed by an incubation at 72 °C for 10 min. Amplification products were separated by electrophoresis in 1.5% (wt/vol) agarose (Promega, Charbonnières, France) gels stained with 0.2 µg/mL ethidium bromide.

All genomic DNAs used in this work were tested for suitability for PCR amplification using primers TubF and TubR (Table 2) in the conditions indicated above.

2.5. Real Time PCR reactions

qPCR reactions were performed in an iCycler iQ5™ Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The PCR thermal cycling conditions were as follows: 95 °C for 4 min, 40 cycles of 94 °C for 45 s, 60 °C for 45 s, 72 °C for 45 s, and 80 °C for 10 s (during which the fluorescence was measured), and final extension at 72 °C for 7 min. Following the final amplification cycle, a melting curve was constructed by measuring the fluorescence continuously when heating from 65 to 95 °C at the rate of 0.5 °C per s. The PCR reaction contained 12.5 µl of of 2X iQ SYBR® Green Supermix (Biorad), 1 µl of each primer (10 µM), 2 µl of template DNA, and sterile bi-distilled water up to a final volume of 25 µl.

To generate the standard curve, a 10-fold dilutions (ranging from 0.5 µg to 0.05 pg) of *F. graminearum* (NRRL 5883) DNA whose concentration was previously determined, were subjected to qPCR under the same conditions described above. Quantification values were automatically determined by the IQ5™ optical system software version 2 (Bio-Rad) and the threshold cycle (Ct) values were then obtained. The standard curve is a plot of the Ct versus log DNA concentration. In all the experiments, appropriate negative controls containing no template were subjected to the same procedure to exclude or detect any possible DNA contamination. Each sample was amplified in triplicate in every experiment. The sensitivity of the real-time assay was validated by continuing the serially (10-fold) diluted DNA up to 0.0005 pg.

2.6. Sequencing and nucleotide sequence accession number

F. culmorum NRRL 25475 genomic DNA was amplified with ZEA-F/ZEA- R. The PCR product was cloned into pCR2.1-TOPO vector (Invitrogen, Cergy Pontoise, France) according to the supplier's instructions. Sequencing of the fragment was performed by MilleGen (Labège, France). The obtained sequence has been deposited in GenBank under accession number: EU362992.1.

2.7. Zearalenone analysis in maize

Determination of ZEA was carried out as described by Schollenberger et al. (2006). Briefly, after extraction with a mixture of acetonitrile and water, sample clean-up was carried out using the Easi-Extract™ Zearalenone immunoaffinity column (IAC) according to the supplier's recommendations (R-Biopharm Rhone Ltd). Identification and quantitation of ZEA was carried out by HPLC.

The HPLC apparatus consisted of a solvent delivery system, with both fluorescence ($\lambda_{ex} = 235$ nm; $\lambda_{em} = 450$ nm) and UV detectors. The analytical column used was a 150×4.6 mm Uptisphere 5 µm C18 ODB fitted with a guard column of 10×4 mm. The column temperature was 30 °C. Kroma 3000 (BIO-TEK) was the data acquisition system.

The mobile phase consisted of HPLC grade acetonitrile (A) and ultrapure water (B). The crude extract was analyzed using a linear elution gradient over 35 min at a flow rate of 0.5 ml/min, starting from 30 to 90% solvent A over the first 30 min, continued by a linear gradient to 90% of (A) in 5 min, and a return to initial conditions over the last 2 min of the run. ZEA was identified by its retention time (29 min) according to a standard (Sigma Aldrich, Steinheim, Germany) and quantified by measuring peak area according to a standard curve. The detection limit was 2 µg/kg.

3. Results

3.1. Primer selection

In this study a *PKS13* involved in ZEA biosynthesis in *F. graminearum* was used as a target in order to design specific primer pair. *PKS13* encoding the enzyme polyketide synthase (PKS) performing sequential condensation of multiple acetate units (Gaffoor and Trail, 2006; Kim et al., 2005; Kroken et al., 2003). The sequence of *PKS13* has been elucidated by (Gaffoor and Trail (2006), Kim et al. (2005) and Kroken et al. (2003). The deduced amino acid sequence of PKS13 was aligned with the other closely related fungal PKSs from the database. A primer pair, ZEA-F/ZEA-R, was designed from two non conserved sequences of PKS13. ZEA-F is positioned at bases 212 to 237 whereas ZEA-R is positioned at the bases 382–403 of the *PKS13*. This set of primers amplified a product of 192 bp in *F. graminearum*.

3.2. Specificity of the PCR reaction

The specificity of the PCR reaction was tested on a diverse range of *Fusarium* strains and other fungal genera commonly associated with cereals. Only DNA of *F. graminearum* and *F. culmorum* was amplified with ZEA-F/ZEA-R and generated a 192 bp PCR product (Fig. 1A). No signal was generated from the none ZEA producing species indicating that the described PCR system is specific for ZEA producing species. The ß-tubulin gene was used as positive control with a fragment of 340 bp obtained in the same PCR conditions for all fungal tested DNA (Fig. 1B).

Since no sequence for PKS13 in *F. culmorum* was available, the PCR amplification product from *F. culmorrum* NRRL 25475 by ZEA-F/ZEA-R has been sequenced. Interestingly the sequencing result showed high similarity with *F. graminearum* fragment of the *PKS13* (Fig. 2).

3.3. Development of the qPCR for the quantification of ZEA producing fungi

The serial 10-fold dilutions of *F. graminearum* DNA ranging from 0.5 µg to 0.05 pg were subjected to qPCR using the designed specific primer ZEA-F/ZEA-R. The quantification relies on measuring the intensity of a fluorescent signal that is proportional to the amount of DNA generated during the PCR amplification. A threshold cycle (Ct) value, corresponding to the PCR cycle number at which fluorescence was detected above threshold, was calculated from the iQ™5 optical system software Bio-Rad).

A standard curve was obtained by plotting the Ct value versus the logarithm of the concentration of each DNA dilution. The linear correlation coefficient of the standard curve was $R^2 = 0.973$ (Fig. 3), demonstrating the accuracy of PCR-based quantification.

Since SYBR® Green I indiscriminately binds to double-stranded DNA, other products in the PCR such as primer dimers may be detected along with the target gene. To verify that the SYBR® Green I dye detected only one PCR product, the samples were subjected to the heat dissociation protocol following the final cycle of the PCR. Dissociation of the PCR reactions consistently produced a single peak, demonstrating the presence of only one product in the reaction (Fig. 4). Interestingly the sensitivity result obtained in this study

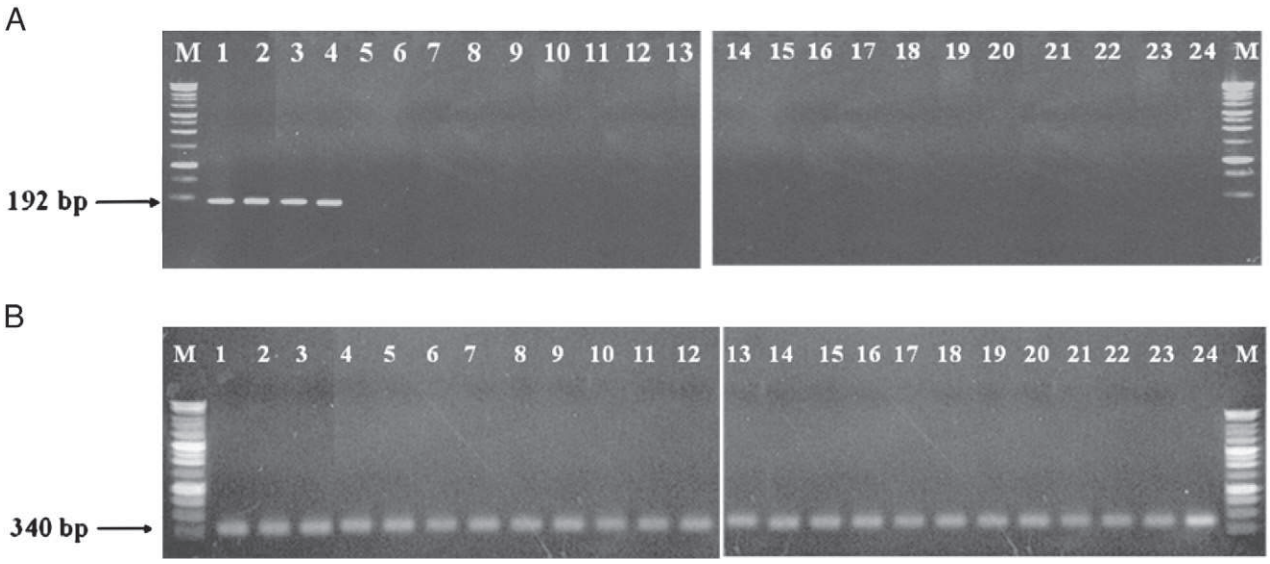

Fig. 1. 1.5% of agarose gel electrophoresis of PCR products with ZEA-F/ ZEA-R (A) and ß-tubulin (B) primers. Lane M, 1 kb DNA ladder (Promega); Lane 1, *F. graminearum* NRRL 5883; Lane 2, *F. graminearum* NRRL 28336; Lane 3, *F. culmorum* NRRL 3288; Lane 4, *F. culmorum* NRRL 25475; Lane 5, *F. proliferatum* NRRL 26191; Lane 6, *F. proliferatum* NRRL 6322; Lane 7, *F. moniliforme* NRRL 13616; Lane 8, *F. verticillioides* NRRL 6442; Lane 9, *F. verticillioides* NRRL 34281; Lane 10, *F. sporotrichioides* NRRL 13440; Lane 11, *F. dlamini* NRRL 13164; Lane 12, *F. poae* NRRL 3287; Lane 13, *F. Poae* CBS 317.73; Lane 14, *Aspergillus parasiticus* CBS 100926; Lane 15, *A. flavus* NRRL 35691; Lane 16, *A. ochraceus* NRRL 5175; Lane 17, *A. westerdijkiae* NRRL 3174; Lane 18, *A. niger* CBS 120166; Lane 19, *A. carbonarius* CBS 120168; Lane 20, *A. sulfureus* NRRL 4077; Lane 21, *Penicillium verrucosum* NRRL 3711; Lane 22, *P. nordicum;* lane 23, *P. citrinum* NRRL 1843; Lane 24, *P. expansum* NRRL 35694.

showed at least 0.005 pg needed be present for a positive reaction with SYBR-Green I (result not shown).

3.4. Application of the qPCR system to quantify F. graminearum and F. culmorum and to estimate ZEA content in maize

The developed qPCR system was used to analyze the occurrence of zearalenone producing *F. graminearum* and *F. culmorum* strains and to predict the toxin which is probably present on maize. For this reason, DNA extractions were performed from thirty-two maize samples (Fig. 3), and subjected to qPCR with the system described above. The amount of *F. graminearum* and/or *F. culmorum* DNA present in an unknown sample was obtained by interpolating its Ct value against the standard curve, and it was expressed in pg DNA/mg of maize. Maize samples also were analysed for zearalenone content by IAC followed by HPLC.

F. graminearum and/or *F. culmorum* DNA content (pg DNA/ mg of maize) was then plotted against ZEA content (ppb) in maize samples. The regression curve showed a positive and good correlation ($R^2 = 0.760$), despite the finding of low levels of DNA in some zearalenone-free samples (Fig. 5). Consequently, results of *F. graminearum* and *F. culmorum* DNA quantification in maize samples could be used for the indirect quantification of ZEA in maize and probably in many raw cereals.

4. Discussion

ZEA is a mycotoxin produced by some species of *Gibberella*/*Fusarium*, especially by *Gibberella zeae* and *F. culmorum* (Stob et al., 1962; Marasas et al., 1984). It is associated mainly with cereal crops, in particular, maize, barley, oats, wheat, rice and sorghum, together with their related products (Kuiper-Goodman et al., 1987). Although this mycotoxin is probably most common in maize, very high levels (11–15 mg/kg) can be found in other cereals, for example barley (Yoshizawa, 1997).

Quantification and identification of mycotoxigenic fungi have traditionally relied on culture methods and morphological classification that require specific expertise and experience particularly in *Fusarium* (López-Errasquín et al., 2007; Jurado et al., 2005; Seifert and Lévesque, 2004; Edwards et al., 2002).

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F.graminearum     1 CTGAGAAATATCGCTACACTACCGACGACGTAGGAATGGCCCAAGCATTCCTGATCTACG
F.culmorum        1 CTGAGAAATATCGCTACACTACCGACGACGTAGGAATGGCCCAGGCATTCCTAATCTACG

F.graminearum    61 CTGTCAGGGCAGGGATACTCCTAAAGTATGTTTCAATCTACCAATGCATCAAGACACACA
F.culmorum       61 CTGTCAGGGCAGGGATACTCCTAAAGTATGTTTCAAATTACCGATGCATCAAGACACACG

F.graminearum   121 TGTATTAACAATATAGATGGGCAAAGTATGAACCAAGCCTCTTGAGTACAGACGAAAATC
F.culmorum      121 TGTATTTACAATCTAGATGGGCAAAGCATGAACCAAGTCTATTGAGTACAGACGAAAATC

F.graminearum   181 AACCTGAGTGGG
F.culmorum      181 AACCTGAGTGGG
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Fig. 2. Nucleotide alignment of the 192 bp amplified region from the *F. graminearum* and *F. culmorum* PKS13. Black shading represent conserved nucleotides, non shaded regions represent nucleotide differences.

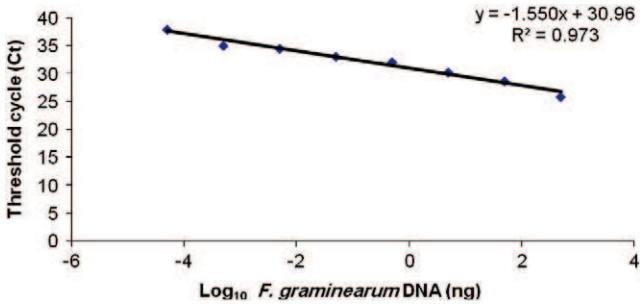

Fig. 3. Standard curve showing the $\log_{10}$ DNA amount (ng) vs. the real-time PCR cycle threshold (Ct) for 10-fold dilutions of *F. graminearum* pure genomic DNA. The assay showed a linear relationship between the DNA amount and Ct with a strong correlation coefficient ($R^2 = 0.97$).

In recent years, several PCR-based techniques have been developed to overcome this problem. These methods have been based mainly on key biosynthetic genes of mycotoxins, the internal transcribed spacer sequence (ITS) and the intergenic spacer region (IGS) of the rDNA (Jurado et al., 2005, 2006; Patiño et al., 2005) as well as from β-tubulin, elongation factor 1 α and the calmodulin genes which provide also highly conserved and variable sequence regions (EL Khoury and Atoui, 2010). However targeting the mycotoxigenic genes is the best way especially where a particular mycotoxin can be produced by a number of species. Regions of homology within mycotoxin biosynthetic gene from the different species can be then used to develop specific primers allowing the detection of these relevant mycotoxigenic species.

There have been reports of collective detection of trichothecene-producing *Fusarium* species with a PCR-based assay based on trichothecene biosynthetic genes (Doohan et al., 1999; Edwards et al., 2001; Schnerr et al., 2001, 2002; Wilson et al., 2004) as well as for fumonisin producing *Fusarium* based on genes in the fumonisin biosynthetic cluster (López-Errasquín et al., 2007). Recently the gene cluster of ZEA biosynthesis has been identified in *F. graminearum* which containing two polyketide synthase gene, *PKS4* and *PKS13* (Lysøe et al., 2006; Gaffoor and Trail, 2006; Kim et al., 2005). In this study we have developed a set of primers, ZEA-F/ZEA-R, and the corresponding PCR assay to detect both *F. graminearum* and *F. culmorum* based on *PKS13*. The assay has been tested on a range of *Fusarium* species as well as on other food relevant fungal species. Positive

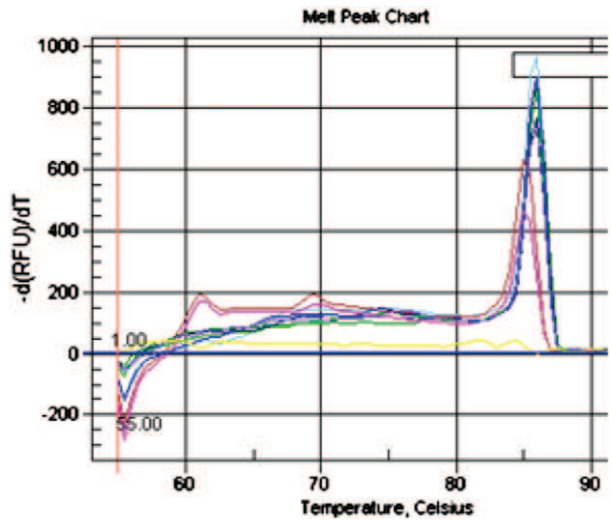

Fig. 4. Melting curve (fluorescence versus temperature) of specific amplificons from the target fragment of *PKS13* at different concentrations. The melting temperature of the target amplicon occurs at 85 °C. No contaminating products are present in the reaction.

results were only obtained from *F. graminearum* and *F. culmorum* producers of ZEA (Fig. 1A). A similar assay has been developed by Meng et al. (2010) but targeting the *PKS4*. Their assay was specific for the zearalenone-producing *F. graminearum*, *F. culmorum* and *F. crookwellense*. Interestingly, in the present work the PCR amplification product from *F. culmorum* by ZEA-F/ZEA-R has been sequenced. Sequencing result showed that the amplified fragment from *F. culmorum* presented high similarity with *F. graminearum* (Fig. 2) within the 192 bp fragment. The developed assay then could be considered as specific for the potential ZEA producing fungi. Sequence differences between *F. graminearum* and *F. culmorum* (Fig. 2) could be used to develop an assay for example, using PCR-RFLP, to differentiate both species.

In the present study, a standard curve ($R^2 = 0.97$) was constructed after qPCR amplification of pure genomic *F. graminearum* DNA using ZEA-F/ZEA-R. This confirmed the linearity of the quantification process between exponential increases in DNA concentration and qPCR threshold cycles (Fig. 3) and showed that it was possible to detect and quantify DNA from *F. graminearum* and/or *F. culmorum* in infected commodities over a range of concentrations, since they have same gene fragment.

The efficiency of PCR quantification methods can be seriously affected by the presence of inhibitors in the food matrix, such as proteinases (Powell et al., 1994) and other compounds naturally present in foods (Mulé et al., 2006). For this reason, testing directly on foods is essential to evaluate the potential application of the developed quantitative real time PCR. In this work DNA quantification of the most relevant ZEA producing fungi *F. graminearum* and *F. culmorum* has been successfully applied in maize. *F. graminearum* and/or *F. culmorum* DNA content was plotted against ZEA content for the analysed 32 maize samples (Fig. 5). A positive and good correlation between DNA quantity and zearalenone was found ($R^2 = 0.760$).

The correlation between DNA content and mycotoxin load has been demonstrated for *Fusarium* and *Aspergillus* species. Schnerr et al. (2002) used the *tri5* gene sequence in a quantitative qPCR to correlate the amount of target DNA with deoxynivalenol contents in wheat samples ($R = 0.9557$). Sarlin et al. (2006) developed PCR assays for trichothecene estimation in barley and malt. They observed high correlation between the *F. graminearum* DNA level and the DON content in north American barley and malt samples ($R^2 = 0.936$), whereas the correlation was not evident with Finnish barley samples which had naturally low DON and *F. graminearum* DNA levels ($R^2 = 0.242$).

Similar results have been reported for other qPCR protocols developed to quantify the ochratoxin A (OTA) in foods. In their results Atoui et al. (2007) and Mulé et al. (2006) showed respectively a positive correlation of $R^2 = 0.81$ and $R^2 = 0.917$ between *A. carbonarius* DNA content and OTA concentration in grape samples. By using real-time PCR, Schmidt et al. (2004) found a positive correlation between the ochratoxin A content and the *A. ochraceus* DNA quantity in green coffee (regression coefficient was $R = 0.55$).

As the goal of the present study was to develop an assay allowing the estimation of ZEA content in maize by the quantification of *F. graminearum* DNA, we can consider that *F. graminearum* DNA content lower than 500 pg DNA/mg of maize could assure good safety. This value corresponds according to the correlation obtained in this study to a value lower than the maximum permitted levels of 350 ppb for ZEA in maize established by the European Union (European Commission Regulation No 1126/2007).

We can conclude that the PCR assays described in this work provides a useful tool for rapid and sensitive detection and quantification of the main ZEA-producing *Fusarium* species which can be readily used to assess the quality of raw material such as maize to be processed into food and feed products. The SYBR Green assay is an advantage for routine analyses of food commodities due to its lower

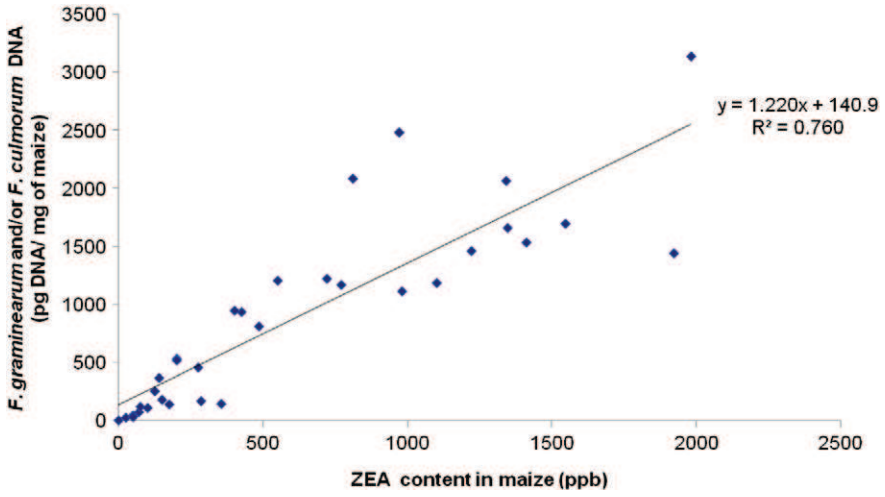

Fig. 5. Correlation of DNA amounts of zearalenone producing fungi (*F. graminearum* and *F. culmorum*) and zearalenone concentrations in 32 maize samples. DNA was quantified using qPCR with primers ZEA-F/ZEA-R and SYBR Green as fluorescent dye.

cost. Moreover qPCR procedures developed in the present study could be carried out in a relatively short time period (4–5 h for DNA extraction and 2–3 h for qPCR).

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