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DETECTION AND QUANTIFICATION OF MYCOTOXIGENIC FUNGI IN MAIZE BY REAL-TIME PCR

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Abstract. The article focuses on real-time PCR detection and quantification of *Fusarium spp*., potential producer of class B fumonisin mycotoxins in mature corn kernels using primers designed to genes FUM1 and FUM6, involved in mycotoxin biosynthesis. Research have shown that the real-time PCR assay can be used for detecting and quantifying potentially mycotoxigenic *Fusarium* in the maize kernels by the genes involved in mycotoxin synthesis. Three pairs of primers specific to genes involved in the synthesis of mycotoxin FB known to contaminate maize and maize products were developed and a pair of primers specific to a conserved *Fusarium beta-tubulin* gene, aimed at detection of most *Fusarium* species. There has been applied quantitative PCR assay for quantification of total *Fusarium spp*. and potentially mycotoxigenic *Fusarium verticillioides*.

Key words: *mycotoxins*, *qPCR*, *Fusarium*, *fumonisin*, *specific primers, contamination, corn kernels*.

Introduction

Mycotoxins are secondary metabolites of filamentous fungi, capable of producing toxic effects to humans and animals. Since they are naturally occurring and widely distributed, their potential presence in food, beverages and feed causes a significant concern worldwide [1].

Current methods of mycotoxin detection include analytical methods which are very precise but laborious and immunological screening methods, which are rapid but expensive and limited by the availability of antibodies [2].

In recent years, there have been considerable efforts to develop alternative methods of detection based on molecular biology techniques. These would determine the amount of mixotoxin-producing fungi rather than the mycotoxin itself. So, for this it would be important to link the amount of mycotoxins in food to the amount of mycotoxin-producing

fungi. Among important mycotoxin-producing fungi are someFusarium species, potent producers of class B fumonisin (FB) mycotoxins which contaminate crops, predominantly maize, all around the world. Within B group fumonisins, fumonisins B1, B2, B3, and B4 are of greatest concern [3].

Their structure includes a linear 20-carbon chain substituted with an amine, two methyls, two tricarboxylate esters, and a C-3 hydroxyl [4].

The genes coding for the enzyme catalyzing fumonisin biosynthesis steps are located in a single FUM cluster. The FB biosynthetic gene cluster consists of 17 transcriptionally coregulatedgenes designated FUM1 through FUM3 and FUM6 through FUM21 (with FUM2 and FUM3 later found to be the same as FUM12 and FUM9, respectively) [5 – 9]. It is suggested that the products of FUM1, FUM8 and FUM6 are responsible for reactions that occur early in fumonisin biosynthesis [3]. According to the proposed pathway for FB biosynthesis, the product of FUM1 gene plays a key role, since it acts at the very early stage of assembly of FB backbone [10].

In this work, we describe the detection and quantification of some potentially mycotoxigenic Fusarium species in maize kernels, by real-time PCR using primers to the genes involved in fumonisin biosynthetic pathway (FUM1 and FUM6).

Materials and methods

We developed three pairs of primers to detect potentially mycotoxigenic *Fusarium* fungi, based on the sequences of FUM1 and FUM6 genes of FB cluster.

Table 1 shows the name of the primers, the genes bearing the sequence of the primers, the organisms bearing these genes, and mycotoxins which biosynthetic pathway uses the products of the genes.

To detect Fusarium spp., we designed a primer pairfbct2 and fbtc3 from the sequence of beta-tubulin gene, a gene not involved in FB synthesis and widely used for molecular taxonomy.

Table 1

Primers developed in this study and genes used for primer development

n/o	Name	Gene	Organism	Mycotoxin
1	mqfumc2 mqfumc3	fumonisin biosynthetic polyketide synthase (FUM1) gene	Fusariumproliferatum, verticillioides, fujikuroi, oxysporum	FB
2	fqfum6ve2 fqfum6ve3	oxygenase (FUM6) gene	Fusariumverticillioides	FB
3	fqprfum62 fqprfum63	oxygenase (FUM6) gene	Fusariumproliferatum	FB
4	fbct2 fbtc3	Fusarium beta-tubulin gene	Fusarium spp.	–

Primers met the requirements for real-time PCR primers: no more than 2 GC pairs on the 3’ end, 5’ and 3’self-complementarity no more than 5.

Primer pairs used in this study are shown in Table 2.

Table 2

Characteristics of the primer pairs used for RT-PCR analysis

Name	Sequence	Template	Length	Start	Stop	Tm	CG%	Self 5'	Self 3'
fqfum6ve2*	TCGCCCTTTGCACCATTGAC	Plus	20	161	180	61.52	55.00	4.00	1.00
fqfum6ve3*	AGCCTGCCGCTTGAACTTTG	Minus	20	339	320	61.80	55.00	3.00	0.00
fqprfum62*	ATCGCCCTCTGCACGATAGA	Plus	20	160	179	60.82	55.00	4.00	0.00
fqprfum63*	TGGGAGGTTGCTCTGAGTGA	Minus	20	282	263	60.47	55.00	5.00	3.00
mqfumc2**	TTCATGAAGCAGTAAGGGCCA	Plus	21	301	321	59.65	47.62	8.00	2.00
mqfumc3**	CATTGATTGCCTCGCCTCTTG	Minus	21	486	466	59.93	52.38	3.00	0.00
fcbt2***	AGCTGTCCAACCCCTCTTACG	Plus	21	901	921	61.78	57.14	4.00	2.00
fcbt3***	CAGCGCGGAAAGAGTGAGC	Minus	19	1104	1086	61.73	63.16	4.00	2.00

**KF889190.1 Fusarium verticillioides isolate 17L oxygenase (fum6) gene, partial cds*

***KF415130.1 Fusarium proliferatum strain ITEM 2287 fumonisin biosynthetic polyketide synthase (FUM1) gene, partial cds_exon2*

****MH521296.1 Fusarium equiseti strain UP-PA002 beta-tubulin gene, partial cds*

Table 2 shows primer name, sequence, orientation, length. It gives its location on the gene (number of start and stop nucleotide), CG content, and 5' and 3' self-complimentarity. PCR conditions were as recommended by CybrGreen producer (Applied Biosystems) – initial incubation at 50°C for 2 minutes, initial denaturation at 95°C for 2 minutes, and alternation of 95°C for 15 sec. and 60°C for 1 minute for 45 cycles. The reactions were performed in 96-well plates (BIORAD) in BIORAD CFX96 touch Real-Time PCR machine. The detection was done at SYBR channel. The asterisks indicate the GeneBank Accession used for primer development. DNA was extracted from mature maize kernels as described [11].

For qPCR standards, fragments were amplified by conventional PCR using the following primer pairs: fcbt2 and fcbt3 for quantification of beta-tubulin gene and fqfum6ve2 andfqfum6ve3 for quantification of FUM6 gene. Then the fragment was visualized on the gel, excised and purified as described [12]. The DNA concentration of the purified DNA fragment was determined by Nanodrop spectrophotometer. Given the fragment size, the copy number per 1 ng of fragment DNA was determined, using an on-line calculator *https://www.thermofisher.com/ro/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna copy-number-calculator.html. Primer efficiency was calculated using qPCR efficiency calculator.*

https://www.thermofisher.com/ro/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/qpcr-efficiency-calculator.html.

Results and discussion

First, the specificity of the primer pairs was checked by using them in a RT-PCR reaction with a mixed sample of DNA purified and pooled from maizekernels of 4 individual plants as a template. Figure 1 shows the amplification plots (Figure 1a) and dissociation curve (Figure 1b) for the primer pairs: 1 – mqfumc2 and mqfumc3; 2 – fqfum6ve2 and fqfum6ve3; 3 – fqprfum62 and fqprfum63.

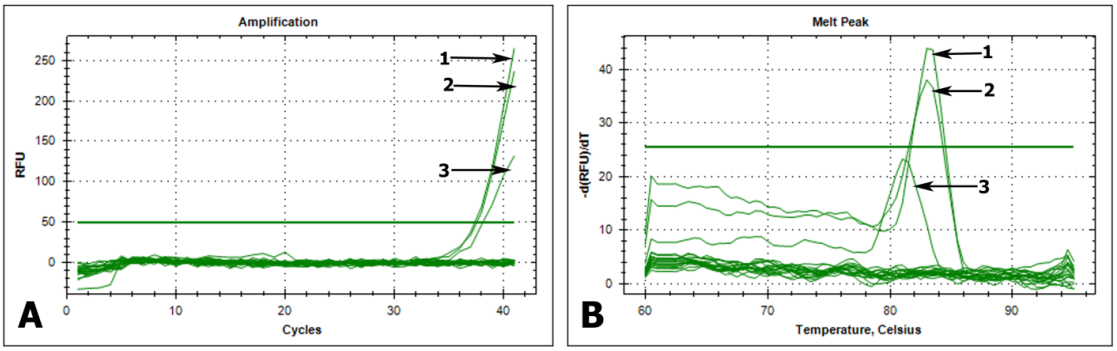

Figure 1. Amplification plot (A) and dissociation curve (B) of the fragment, amplified by primer pairs mqfumc2 and mqfumc3 (1), fqfum6ve2 and fqfum6ve3 (2), fqprfum62 and fqprfum63 (3).

As one can see, the primers could successfully amplify the target sequence, and a single pronounced peak in the dissociation chart shows the uniformity of the amplified fragment, indicating primer specificity.

Figure 2 shows the amplification plots (Figure 2a) and dissociation curve (Figure 2b) for the primer pair fbct2 and fbtc3. This pair of primer was designed to detect a conserved sequence of Fusariumbeta-tubulin gene, not involved in the synthesis of mycotoxins. Thus, they were designed to detect all Fusarium species, mycotoxigenic or not.

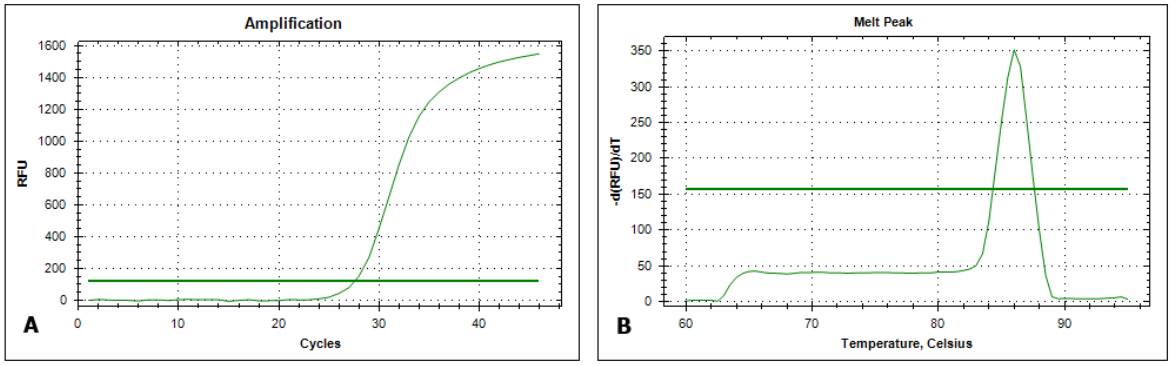

Figure 2. Amplification plot (A) and dissociation curve (B) of the fragment, amplified by primer pair fbct2 and fbtc3.

Then, primer pair 2 (fqfum6ve2 and fqfum6ve3) was used for quantifying FUM6 gene in maize samples. For this, serial dilutions of DNA standard with known copy number was analyzed by Real-time PCR, the Ct values were recorded and used to build a standard curve. At the same time, DNA of kernels from four individual plants (samples 1, 2, 3, 4) was tested for the presence of FUM6 gene with this pair of primers. These results are shown in Figure 3. Calculated efficiency of primers for the slope is 93%.

One out of four maize samples (sample 1) was tested positive for the FUM6 gene. Using the relationship, we could calculate the number of copies of FUM6 gene in this sample. The average Ct value of 3 technical replicas was 33,37 which corresponds to about 780 copies per 20 ng of input DNA. This is shown by a star in the graph (Figure 3).

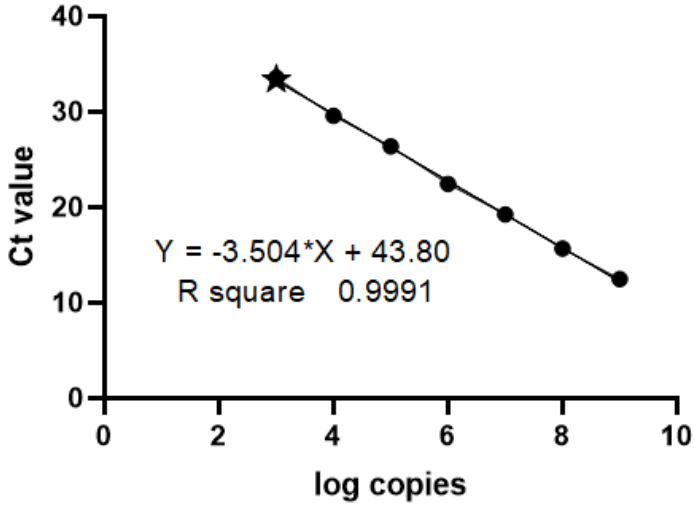

Figure 3. The relationship between the initial copy number of the template in the reaction and the Ct value for primer pair 2 (fqfum6ve2 andfqfum6ve3) to FUM6 gene. **The star indicates the log copy number/Ct value of the FUM6 gene in the maize sample 1 which gave positive signal with this pair of primers.*

Next, we wanted to test the same samples using primers to *Fusarium* spp. So, serial dilutions of DNA standard with known copy number was analyzed by Real-time PCR, the Ct values were recorded and used to build a standard curve. Calculated efficiency of primers for the slope is 105%.

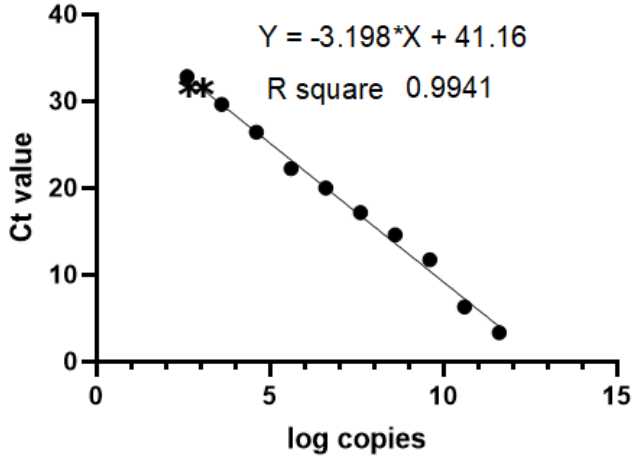

Figure 4. The relationship between the initial copy number of the template in the reaction and the Ct value for primer pair 4(fcbt2 and fcbt3) to Fusarium beta-tubulin gene. **The asterisks indicate the log copy number/Ct value of the fusarium beta-tubulin gene in the maize samples 1 and 2 which gave positive signal with this pair of primers.*

Two out of four maize samples were tested positive for Fusarium beta-tubulin gene. Using the relationship, we could calculate the number of copies of this gene in these samples. The average Ct value of 3 technical replicas was 32,12 for one sample and 32,34 for the other positive sample. This corresponds to about 650 copiesper 20 ng of input DNA. Summarized data of the qPCR analysis of four maize samples, are shown in Figure 5.

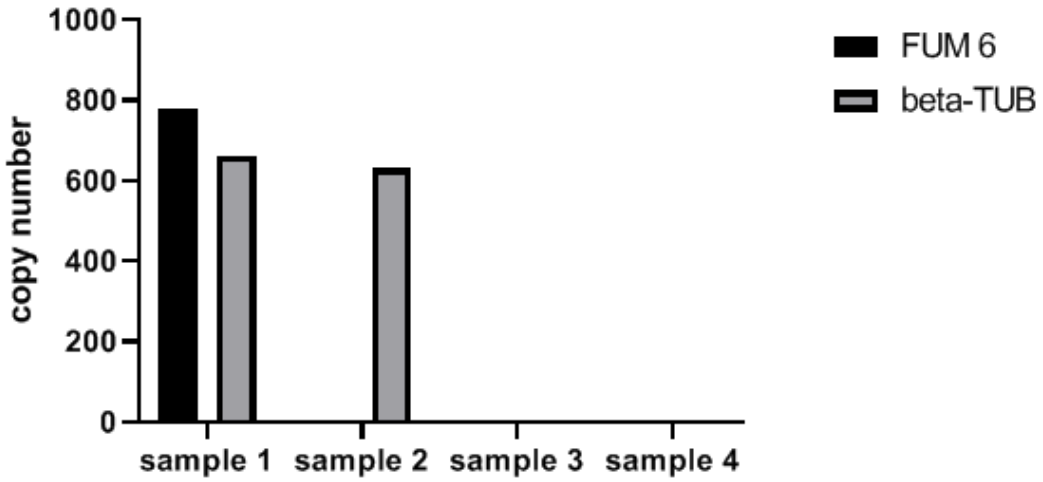

Figure 5. Summarized data of the qPCR analysis of four maize samples.

As one can see, in the first sample both pairs of primers gave a positive signal, meaning that both FUM6 of Fusarium verticillioides (gene involved in mycotoxin synthesis) and Fusarium beta-tubulin (gene not involved in mycotoxin synthesis) genes are present in the sample. The copy number of the detected fragments is very similar, so, assuming that both genes are present in the genome in a single copy, one can suppose that all Fusarium present in the first maize sample is Fusarium verticillioides containing FUM6 gene, and thus is potentially mycotoxigenic. The second maize sample was positive only for Fusarium beta-tubulin gene, but not for Fusarium verticillioides FUM6. So, this sample is probably contaminated with a different Fusarium species. The other two samples (3 and 4) were not tested positive for either of the genes, meaning that probably the samples were not infected with Fusarium.

Conclusions

As a result of this work we developed three pairs of primers specific to genes involved in the synthesis of mycotoxin FB known to contaminate maize and maize products. We also developed a pair of primers specific to a conserved Fusarium beta-tubulin gene, aimed at detection of most Fusarium species. We set the quantitative PCR assay for quantification of total Fusariu*m* spp. and potentially mycotoxigenic Fusarium verticillioides. We tested four maize samples and compared the amount of Fusarium spp. and Fusarium verticillioides in those samples. The real-time PCR assay can be used for detecting and quantifying potentially mycotoxigenic Fusariumin the maize kernels by the genes involved in mycotoxin synthesis. This can be a step towards establishing a possible correlation between the amount of mycotoxingenic pathogen and amount of mycotoxin in food sample.

Acknowledgment

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