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POISSON DISTRIBUTION-BASED CONVENTIONAL PCR PROTOCOL FOR QUANTIFICATION OF PATHOGENIC FUNGI IN MAIZE

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Rezumat

Studiul actual își propune optimizarea și aplicarea protocolului de PCR convențională bazat pe principiul testului digital pentru cuantificarea fungilor patogeni din porumb. Reacia PCR convențională bazată pe distribuția Poisson este o metodă eficientă și simplă din punct de vedere tehnic pentru analiza cantitativă a agenților patogeni ai plantelor. Procedeul nu necesită reactivi specifici sau echipamente de laborator sofisticate. Spre deosebire de qPCR, nu este necesară nicio curbă standard pentru cuantificarea numărului de copii per probă. Această metodă a permis cuantificarea mai multor agenți patogeni fungici toxici și netoxici în plantele de porumb și dezvăluie impactul genomului asupra cantității de ADN fungic din organismul gazdă. În general, cantitatea fungică a fost cea mai mică în 'MK01' și 'KU123' și cea mai mare în 'CP137'. A fost notificată o cantitate mare de ADN fungic și pentru „'CP148'. Pentru 'B73' cantitatea de ADN fungic a fost între aceleași valori pentru 'KU123' și 'CP148'.

Cuvinte cheie: PCR, porumb, distribuția Poisson, *Fusarium, Penicillium, Aspergillus*

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Introduction

Quantitative analysis is essential for monitoring fungal propagation in host-plant and dynamics of plant diseases. Maize is attacked by a wide specter of fungal pathogens, main causal agents of kernel deterioration and corn diseases are comprised by *Fusarium, Aspergillus* and *Penicillium* genera[1–5]. The major maize ear rot diseases include *Fusarium* ear rot (main causal agents – *F. verticillioides, F. proliferatum, F. subglutinans*) and *Gibberella* ear rot, caused by *F. graminearum* species complex [6]. *Aspergillus* and *Penicillium* species are well-known post-harvest pathogens that induce corn deterioration during storage [7, 8]. These fungal genera require special attention as they comprise species that produce dangerous mycotoxins: fumonisins, patulin, aflatoxins and others [2, 8–10].

Among molecular methods, real-time PCR is considered the most effective and robust – gold standard, for quantifying plant pathogens and was successfully used for solving a number of problems in phytopathology [11–14]. However, qPCR implies using of expensive thermal cyclers and supporting real-time PCR instruments and reagents.

Therefore, for some types of practical issues is reasonable the use of more cheap quantitative methods, especially when it concerns low-income laboratories.

The main alternative for real-time PCR is based on digital assays that antedated the quantitative PCR. Combination of limiting dilution, conventional PCR and Poisson statistics was originally used for quantitation of initial DNA targets in a sample [15]. Afterwards the principle of digital assay-based PCR was improved and optimized, which resulted in developing the digital PCR and droplet digital PCR (ddPCR)[16–20].The main difference between conventional or qPCR and digital PCR is that in digital PCR the reaction is divided into many reactions such that each reaction contains a single molecule of interest or not as the case might be, and a digital (all or none) signal is obtained. It allows determining the "absolute quantification" of target nucleic acid sequences by counting positive amplification signals derived from amplification of a single DNA template from minimally diluted samples. In contrast to qPCR, the quantification of nucleic acid in dPCR does not base on the CT values, standard curves, and internal controls. Currently, dPCR is a widespread method for the nucleic acid measurement due to the lower cost; it has been used extensively in the diagnostic laboratory for the detection of genetically modified organisms, foodborne pathogens and others[21].

Currents study aims to optimize and apply a conventional PCR protocol based on digital assay principle for quantification of pathogenic fungi in maize.

Materials and methods

Plant material and DNA extraction. Leaves, anthers, silk and mature kernels of several maize genotypes ('CP137', 'CP148', 'MK01', 'KU123', and 'B73') were collected for the analysis from experimental fields of the Institute of Genetics, Physiology and Plant Protection. Total DNA was extracted using a combination of SDS-method with subsequent CTAB-purification [22–24]. DNA was quantified using commercial DNA molecular weight markers (Thermofischer Scientific).

Primers. Specific primers were designed using fungal genomic sequences presented in GenBank database (tab. 1). Primers for *Penicillium chrysogenum* were designed based on housekeeping β-tubulin gene sequence, while primers for identification of toxigenic *Aspergillus* and *Fusarium* species were constructed using sequences for gene clusters associated with mycotoxin synthesis.

Digital assay. A sample of extracted DNA was subjected to a series of dilutions (1:10, 1:20, 1:50 and 1:100). A final volume of 300 µl of each series was partitioned in 30 reaction tubes and used for amplification. Two subsequent amplifications were performed. A bulk nested-PCR to screen the samples that contain fungal DNA was followed by a conventional PCR with sample DNA for quantification of target sequences using Poisson distribution.

Quantification of fungal DNA copy number in maize samples was performed using Poisson statistics from the proportion of negative endpoints: m= -n* ln(E), where m is number of targets per partition, (E) - percentage of empty samples (no amplification), n – number of samples in a reaction series.

Amplification. PCR was performed in 25 µl of mix containing: 66 mM Tris-HCl (pH 8.4), 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.1 % Tween 20, 7% glycerol, 0.01 µg BSA, 0.2 mM of each dNTPs, 1.25 U Taq DNA polymerase (Thermo Fisher Scientific), 5 pM of each primer and 10 µl of diluted DNA template. Negative control with water as template was included to detect possible contamination.

Table 1. Primers designed for PCR-identification of several fungal pathogens

Primer	Sequence	Fungi	Target sequence (FASTA)
ftri8gr1(F)	CTTCCGGTAATGTTTCTCGTCACT	*Fusarium graminearum*	MH514940.1 *Fusarium graminearum* isolate 23-4 Tri core gene cluster, complete sequence [25]
ftri8gr4(R)	CGCTGCTGAGGGTTTTACCAT		
fqtri8gr2(F)	CTCGTCACTTCCTTGATGACACA		
fqtri8gr3(R)	GGGGGCCGACATTCACTTC		
afap1(F)	CTTTGTTCGGTAGTGCCATCTTGA	*Aspergillus flavus*	FJ877830.1 *Aspergillus flavus* strain IC289 O-methyltransferase A (aflP) gene, partial cds; and aflP-aflQintergenic spacer, partial sequence[26]
afap4(R)	GCCATAGCACATATTCTCCAACCT		
aqfap2(F)	GTGTCGGGTGTGCCTATTTAACC		
aqfap3(R)	AAGGCTTTCGGTCGGTTGATG		
apap1(F)	TTGCTCGGTAGTGCCATGTT	*Aspergillus parasiticus*	DQ390914.1 *Aspergillus parasiticus* strain IC73 O-methyltransferase A (aflP) gene, partial cds; and aflP-aflQintergenic region, genomic sequence[27]
apap4(R)	GGCTTCCATAACACATATTCTCCAA		
aqpap2(F)	CCGCGAAAGAACAAACAGAGA		
aqpap3(R)	AACACATATTCTCCAACTTTCTTGCT		
pchbt1(F)	GTTGCTAACTGGATTACAGGCAAAC	*Penicillium chrysogenum*	GQ498281.1 *Penicillium chrysogenum* strain NRRL_1950 β-tubulin (benA) gene, partial cds[28]
pchbt4(R)	CACCGCTGGCCTAGATTGTC		
pqchbt2(F)	TGATGGGGATTCTGGTGGATCA		
pchbt3(R)	CCGCTGGCCTAGATTGTCAAA		

Nested-PCR protocol included in round I 1 cycle denaturation at 95°C for 3 min, followed by 30 sec denaturation at 95°C, annealing at 60°C for 30 sec, extension at 72°C for 30 sec for 30 cycles. Second round included 30 sec denaturation at 95°C, annealing at 60°C for 30 sec, elongation at 72°C for 30 sec for 30 cycles.

Conventional PCR protocol included 1 cycle denaturation at 95°C for 3 min, followed by 30 sec denaturation at 95°C, annealing at 60°C for 30 sec, extension at 72°C for 30 sec for 45 cycles.

The products of amplification were separated in 1.5% agarose gel at the 6V/cm in a 1xTBE migration buffer (pH 8.0) with ethidium bromide, viewed in the UV (302 nm)

and photographed. Amplicon length was estimated using 100 bp DNA ladder (Thermo Fisher Scientific) and GelAnalyzer2010 software.

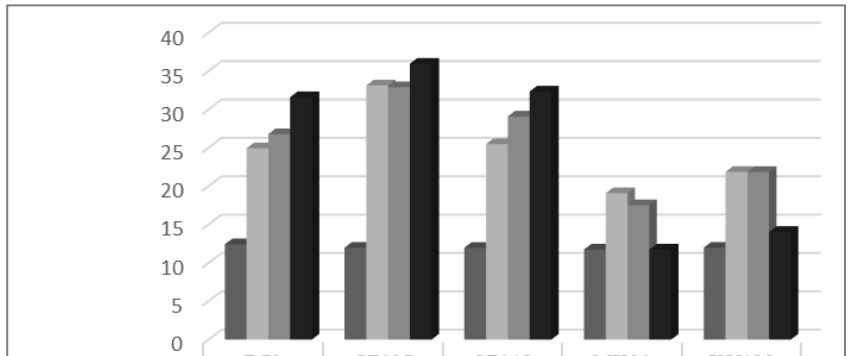
Statistical analysis was performed using one-way ANOVA test, $p<0.05$ (STATISTICA software package, TIBCO Software Inc.)

Results and discussions

Digital assays are based on partitioning statistics, when initial sample volume is divided in n-partitions and the number of DNA copies depends on the number of partitions and probability of negative or positive endpoints, governed by binomial and Poisson distribution.

The main objective of the digital assay for fungal pathogens' quantification is to obtain a certain number of negative endpoints in the amplification, which makes target sequence quantification possible. Low-scale dilution may produce a high percentage of positive endpoints, while highly diluted sample DNA may result in losing target sequences in case of identification of rare pathogens. In this study, optimum percentage of negative endpoints was obtained with the use of 50-fold diluted sample DNA. Tenfold and 20-fold dilutions gave high percentage of positive endpoints (above 90%). A highly diluted sample DNA (100-fold) resulted in disappearance of *F. graminearum* in a series of amplifications. Therefore, 50-dilution of initial DNA concentration of 5ng/µl was stated as optimal for fungal DNA quantification.

First, a qualitative bulk amplification using nested-PCR was performed to detect positive samples. All positive samples were subjected to a series of dilutions and used for quantitative analysis via conventional PCR. After conventional PCR all negative endpoints were calculated, and target sequence quantity was computed using formula (1). The final value is presented in absolute number of fungal DNA sequences per sample (fig. 1).



	B73	CP137	CP148	MK01	KU123
P. chrysogenum	12.43	11.98	11.96	11.75	11.97
A. parasiticus	24.93	33.13	25.48	19.08	21.86
A. flavus	26.78	32.9	29.09	17.51	21.86
F. graminearum	31.6	35.96	32.34	11.74	14.04

Figure 1. Mean number of fungal DNA copies per sample for analyzed maize genotypes

Mean values of fungal DNA copies per sample differed among pathogens' species as well as between maize cultivars. Genotype impact on overall fungal DNA quantity in maize samples was significantly high ($F=46, 2$; $p<0.001$). There was no significant

statistical difference in *P. chrysogenum* quantity between 'CP137', 'CP148', 'MK01', 'KU123', and 'B73' (F=0.225564, p=0.921525). This fungus is considered a week pathogen and is commonly associated with maize kernels' deterioration during storage.

However, for other three mycotoxigenic pathogens the results were different. Concentration of *F. graminearum* was significantly higher in 'CP137', 'CP148' and 'B73' compared to 'MK01' and 'KU123' ($p<0.05$). Same pattern was observed for *A. flavus* and *A. parasiticus*, the highest values of fugal DNA per sample was computed for 'CP137' and 'CP148'. There was no significant difference ($p<0.05$) between *A. flavus* and *A. parasiticus* DNA copies per sample, their concentration being closely similar.

Overall, fungal quantity was lowest in 'MK01' and 'KU123' and highest in 'CP137'. High quantity of fungal DNA was also computed for 'CP148'. For 'B73' the fungal DNA quantity was in between the same values for 'KU123' and 'CP148'.

Conclusions

Quantitative analysis of A. flavus, A. parasiticus, F. graminearum and P. expansum in maize plants of 'CP137', 'CP148', 'MK01', 'KU123' and 'B73' was performed.

The highest mean DNA quantity was calculated for A. flavus (25,63 copies per sample), the lowest – for P. chrysogenum (12,02 copies per sample).

The highest mean fungal DNA quantity was calculated for 'CP137' (28,49 copies per sample), the lowest – for 'MK0'1 (15,02 copies per sample) and 'KU123' (17,43 copies per sample).

There was no significant difference in quantity of P. chrysogenum for maize plants of the studied genotypes.

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