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EVALUAREA REZISTENȚEI A PLANTELOR DE PORUMB LA SPECIILE DE FUSARIUM PRIN METODA PCR

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Contaminarea câmpurilor de porumb cu speciile fungice de Fusarium duce la scăderea volumului și calității de roadă. În lucrarea dată, trei genotipuri de porumb la fazele de mătăsare, lapte-ceară și maturitate fiziologică au fost analizate pentru prezența infecțiilor mixte de F. verticillioides, F. oxysporum, *F. avenaceum*. Infecțiile mixte asimptomatice de *F. oxysporum*, *F. verticillioides*, *F. avenaceum* au fost identificate în toate mostrele de Ku123 și în unele mostre ale genotipului RF7. Genotipul MK01 a fost infectat doar cu *F. oxysporum*. Estimarea numărului de copii prin metoda PCR cu aplicarea diluțiilor seriale a demonstrat că genotipul Ku123 a fost cel mai susceptibil la infecție.

***Cuvinte-cheie**: speciile de Fusarium, plante de porumb, PCR, rezistență.*

EVALUATION OF MAIZE PLANT RESISTANCE TO FUSARIUM SPECIES BY PCR

Fungal contamination of maize fields with *Fusarium* species leads to yield loss and affects grain quality. In this investigation, maize of three genotypes Ku123, RF7, MK01 at the silking, dough and physiological maturity stage were analyzed for mixed contamination with *F. verticillioides*, *F. oxysporum*, *F.avenaceum*. Asymptomatic mixed infection with *F. oxysporum*, *F. verticillioides*, *F. avenaceum* was detected in all samples of Ku123 and in some samples of the genotype RF7. The genotype MK01 was infected only with *F. oxysporum*. PCR estimation of the number of copies of *Fusarium spp* by serial dilutions demonstrated that genotype Ku123 is the most susceptible to the infection.

***Keywords:** Fusarium species, maize plants, PCR, resistance.*

Introduction

Fungal contamination of maize fields with *Fusarium* species leads to yield loss and affects grain quality. Main factors that define grain infection rate are species composition of *Fusarium* fungi, and grade of host-genotype resistance. Infections with Fusarium spp. are serious risk to the quantity and quality of maize products, which in case of mycotoxin contamination may cause significant adverse effects on the health of humans and animals [1-4]. *Fusarium* infections may lead to the appearance of symptoms but can also cause symptomless infection. Plant disease monitoring and early diagnosis are essential to prevent pathogen dissemination. PCR-based assays of molecular diagnostics allow rapid and precise pathogen identification, including cases of mixed contamination, differentiation of species causing similar symptoms [5, 6]. The sensitivity and specificity of detection of the target pathogen are significantly improved when using nested-PCR, by performing a second PCR round using the internal primers for the amplification product [7-9]. Efficiency for detection of fungi is based on the primer specificity. The sequence of a partial fragment of the translation elongation factor 1-alpha (TEF-1α) gene has sufficient variability for differentiation at the level of Fusarium genus and is widely used to create specific primers for individual species [10]. The use of nested-PCR to assess the number of copies of pathogenic DNA can also be a method for estimating the resistance of maize plants to infection with *Fusarium spp*. Serial dilution of DNA targets with PCR has been used by many researchers [11-13]. This necessarily requires that the PCR protocol is optimized for sensitive detection of a single, or a known number, of target molecules.

The aim of the current research is: a) PCR identification of Fusarium species in DNA samples isolated from maize cobs at different stages of development (silking, dough and physiological maturity); b) determining the number of copies of Fusarium spp. DNA in maize lines with different degrees of resistance to Fusarium by the method of serial dilutions.

Materials and methods

Maize samples of Ku123, RF7, MK01 lines were collected from the experimental field of the Institute of Genetics, Physiology and Plant Protection. Plants were randomly collected at several physiological stages of growth (silking, dough and physiological maturity). DNA was isolated from 1 gram of corn kernels, mainly as described in ISO 21571:2005 [14] successively using the methods "Preparation of PCR-quality DNA using polyvinyl-pyrrolidone (PVP)-based DNA extraction methods", (Annex A2) and "CTAB-based DNA extraction methods", (Annex A3).

Nested-PCR for *Fusarium* fungi identification was performed using primers designed to amplify a partial sequence of the translation elongation factor 1-alpha (Tab.1). For primer design, we use the program Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), found on NCBI site.

Both rounds of nested PCR were performed in 25 µl of PCR mixture 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 DNA template. Nested-PCR protocol included in first round - 1 cycle of denaturation at 95°C for 3 min, followed by 1 min denaturation at 95°C, annealing at 60°C for 1min, extension at 72°C for 1min, 30 cycles. Second round included 1min denaturation at 95°C, annealing at 60°C for 1min, elongation at 72°C for 1min for 30 cycles. PCR products were separated in 1,5% agarose gel electrophoresis (6V/cm), stained in with ethidium bromide, viewed in the UV (302 nm). Product length was estimated using DNA ladder 100 bp (Thermo Fisher Scientific).

The number of copies of pathogen DNA was determined using the method of serial dilutions to a concentration when only one molecule remains in the sample, and after the next dilution, the amplification signal is absent.

Table 1. Primers used for nested-PCR identification of Fusarium species.

Pathogen	Primer	Primer sequence 5'- 3'	Amplicon bp	GenBank accession numbers
Fusarium spp.	fc2	CTACCAGTGCGGTGGTATCG	431	ON844090.1 Fusarium commune strain DAR 85434 (TEF-1α) gene, partial cds
	fc6	ACATACCAATGACGGTGACATAGT		
	fc3	CCATCGAGAAGTTCGAGAAGGTT	300	
	fc4	CCCAGGCGTACTTGAAGGAA		
F. oxysporum	fo1	ATCTGCCATCGTCAATCCCG	576	OQ181366.1 Fusarium oxysporum isolate CF40-21 (tef-1a) gene, partial cds
	fo6	GACCGGGAGCGTCTGAGT		
	fo2	TCAATCCCGACCAAGACCTG	329	
	fo4	ACGTGACGACGCACTCATT		
F. avenaceum	fa2	CTCCCATCGATTCCCACGAC	192	KY365600.1 Fusarium avenaceum strain FOEP 40.11085 (tef1) gene, partial cds
	fa5	GTGACTGCAAGACATAGTGCG		
	fa3	CGACTCGCTCCCTCATTCG	140	
	fa4	GTTTTGTGGGAACAGGGCAAG		
F. verticillioides	fv1	GATGAGCTTATCGGCCATCGT	579	OM812702.1 Fusarium verticillioides isolate MFvKA-42 (TEF 1 alpha) gene, partial cds
	fv6	CCGGGAGCGTCTATGTGATG		
	fv2	ATCGTAAACCCGGCCAAGAC	310	
	fv5	GAGGTTGTGGAATGGGAGAGG		

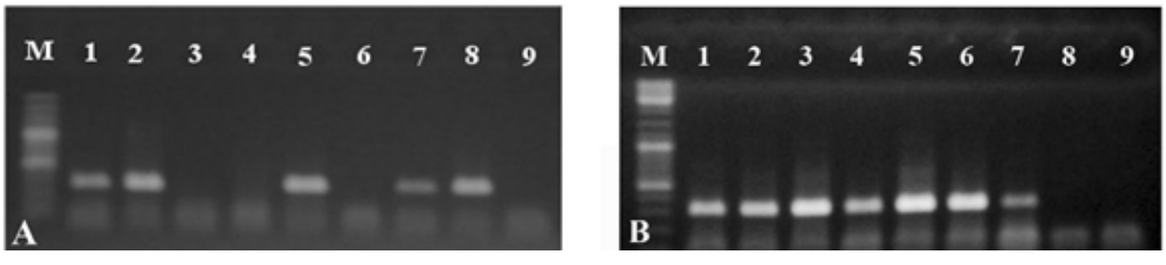
Results and Discussion

Samples of the cobs at the silking, dough and physiological maturity stage of Ku123, RF7, MK01 lines were analyzed. Primarily, DNA samples were analyzed for the presence of *Fusarium spp*. In the cob samples at the silking stage *Fusarium spp*. were not detected in any of the studied genotypes. Plant infection starts to develop at the dough stage. In case of positive signal (synthesis of a specific amplicon – 300 bp) samples were further analyzed for specification of *Fusarium* species. Nested-PCR detection of *F. oxysporum* (synthesis of a specific amplicon – 329 bp) in cob samples at the dough stage (top and base parts of the cobs) in four DNA samples of MK01 and Ku 123 lines is shown on Fig. 1. Amplicon specific for Fusarium spp. was not detected in the one sample of both MK01 and Ku123 maize lines.

Fig. 1. Nested - PCR analysis of F. oxysporum in the cobs at the dough stage:

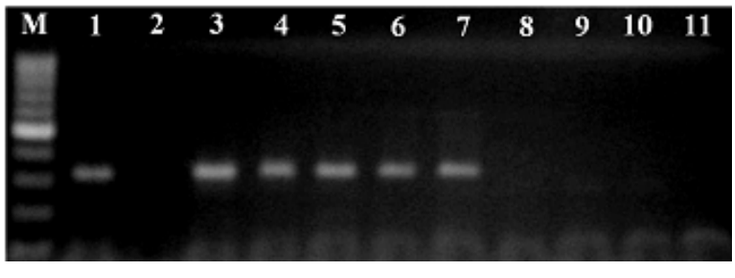
A - genotype MK01. Lanes 1-4 DNA samples of top part of the cobs, 5- 8 DNA samples of base part of the cobs; 9 – negative control, M-100 bp marker.

B - genotype Ku123. Lanes 1-4 - DNA samples of top part of the cobs, 5-8 DNA samples of base part of the cobs; 9 - negative control, M-100bp marker.



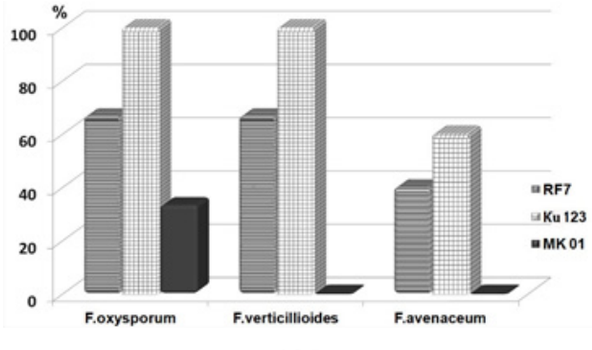
PCR detection of *F. verticillioides* (synthesis of a specific amplicon – 310 bp) in grain samples of Ku 123 and RF7 lines at the dough stage is shown on fig. 2.

Fig. 2. Nested-PCR analysis of F. verticillioides in the samples at the dough stage. Lanes 1-5 DNA samples of genotype Ku123, 6-10 DNA samples of genotype RF7; 1- negative control, M-100bp marker.



The distribution of the mixed infection of Fusarium fungi in corn cobs of Ku123, RF7, MK01 lines at the dough stage is presented on fig.3.

Fig. 3. Distribution of mixed Fusarium fungal infection in corn cobs at the dough stage in lines Ku123, RF7, MK01.



The analyzed MK01, RF7 and Ku123 lines showed different degree of infection with different *Fusarium* species at the dough stage. Asymptomatic mixed infection with *F. oxysporum*, *F. verticillioides* и *F. avenaceum* was detected in all samples of Ku123 and in some samples of the RF7 genotype. The genotype MK01 was infected only with *F. oxysporum*, with infection rate of 30%, compared to the infection levels with the same pathogen of RF7 (60%) and Ku123 (100%) lines.

Another approach to determining the resistance of maize genotypes to *Fusarium* infection is the determination of the pathogen DNA copy number by the method of serial dilutions. Serial dilutions are performed up to the concentration when a single target DNA sequence is present in the sample, while the following dilution shows no signal, and thus no nested-PCR amplification of the fragment is conducted.

This means that, knowing the dilution factor, it is possible to calculate how many copies of the analyzed DNA were present in the undiluted sample. For nested-PCR analysis we used primers specific to *Fusarium spp* and a mixture of 5 DNA samples for each of the two lines of MK01 (fig.4) and Ku123 (fig.5) lines at dough and physiological maturity stages.

Fig. 4. DNA dilution of mixed samples of line MK01. A -ten sequential dilutions 1:2 of DNA samples at dough stage, starting from $2x10^{-1}$ to 10^{-3}fold; B- ten sequential dilutions 1:2 of DNA samples at physiological maturity stage, starting from $2,5x10^{-2}$ to $6,4x10^{-5}$ fold. Numbers in figures A and B indicate the last sample dilution, when the amplification signal is still detected.

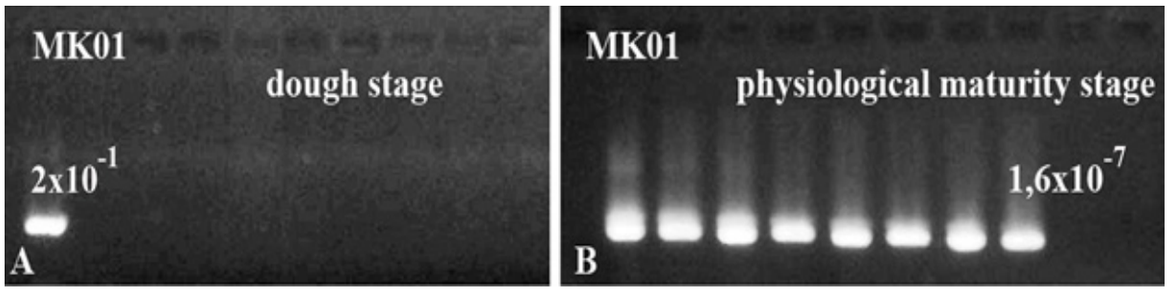
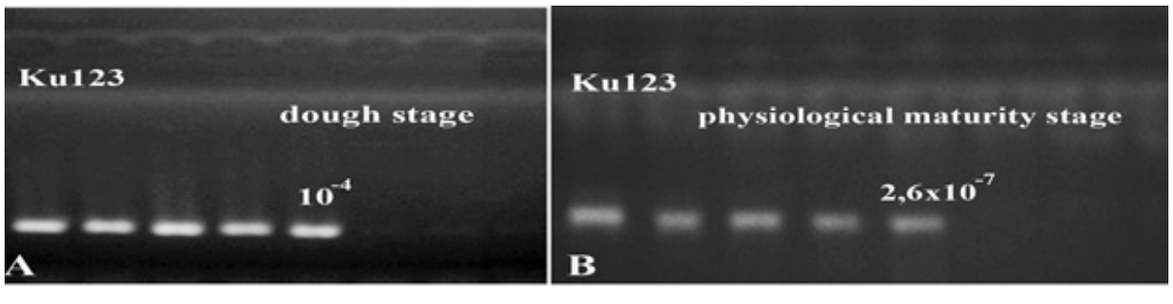


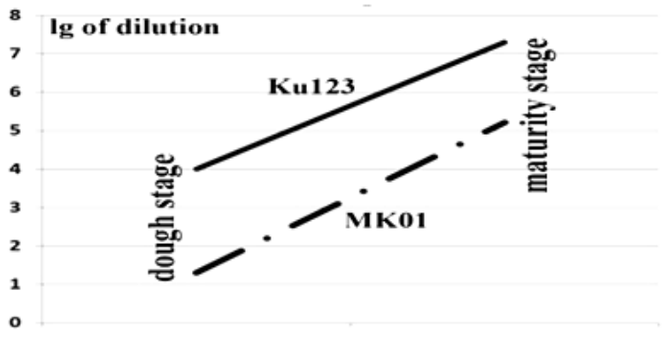
Fig. 5. DNA dilution of mixed samples of line Ku 123. A – eight sequential dilutions 1: 2 of DNA samples at dough stage, starting from $6,4x10^{-2}$ to $8,16x10^{-4}$ fold; B – eight sequential dilutions 1:2 of DNA samples at physiological maturity stage, starting from $1,6x10^{-6}$ to $2,1x10^{-8}$. Numbers in figures A and B indicate the last sample dilution, when the amplification signal is still detected.



As a result, it was shown that at least 20 copies of the DNA of the pathogen are present in 1 g of MK01 sample at the dough stage and at least $1,6x10^5$ copies at the physiological maturity stage. In case of Ku123 line, at least 10^4 pathogen DNA copies are present in 1 g of the sample at dough stage, and at least $2,6x10^7$ copies at the physiological maturity stage.

The concentration of the DNA of *Fusarium spp*. increases with maturation and depends on the resistance of maize genotype. The Ku123 genotype showed a maximal amount of infected kernels, thus it is the most susceptible to the infection, the genotype MK01 demonstrated to be the most resistant. The graph of the changes in values of the common logarithm of DNA dilution factor for both maize lines at two stages of development is presented on fig. 6.

Fig. 6. Lg of DNA dilution of the samples MK01 and Ku 123 in the dough and physiological maturity stages.



Therefore, both percentage of infection and the amount of pathogen's DNA analysis in kernels of cobs at dough stage using nested-PCR can be suggested as a method for evaluating maize resistance to *Fusarium* species at early stages of development. PCR-diagnostics of phytopathogens can be important for preventing the spread of the *Fusarium* pathogens, which are known producers of mycotoxins, can be present in infected grain and eventually lead to the loss of yield and grain quality. Fusarium infection can lead to manifestation of symptoms, but also can cause asymptomatic infection. Thus, the described approaches can be used for deeper understanding of the process of infection and screening of maize genotypes based on the susceptibility to *Fusarium* fungi, as well as for evaluation of the resistance of these genotypes.

Conclusions

Molecular analysis demonstrated that infection of corn cobs begins to develop at the dough stage, however, the analyzed genotypes Ku123, RF7, MK01 showed different degrees of infection with *F. oxysporum*, *F. avenaceum* and *F. verticillioides*. The genotype MK01 was infected only with *F. oxysporum*, with infection rate of 30%, compared to the infection levels with the same pathogen of RF7 (60%) and Ku123 (100%) lines. Thus, nested-PCR analysis of the cob at the dough stage can be proposed as a method for early diagnosis of the resistance of maize genotypes to Fusarium pathogens. Another approach to evaluating the resistance of maize genotypes to Fusarium infection is the determination of the pathogen DNA copy number by the method of serial dilutions. In this case, the evaluation of maize lines MK01 and Ku123 to *Fusarium spp*. was carried out both at dough stage and physiological maturity stage. In the present study, the MK01 line proved to be more resistant to the fungal infection, compared to Ku123 line.

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