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IDENTIFICATION OF MYCOTOXIN-PRODUCING SPECIES OF FUSARIUM IN TOMATO ONTOGENESIS

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Summary. The results of molecular identification of toxigenic *Fusarium spp*. in local tomato varieties at some vegetative phases are presented. *F. oxysporum, F. verticillioides, F. proliferatum, F. avenaceum, F. equiseti, F. solani* were found at different stages of tomato development. Sequences of FUM gene cluster involved in the synthesis of Fumonisin B1 and Polyketide synthase (PKS13) gene sequences involved in the synthesis of Zearalenone were revealed in some tomato varieties infected by certain toxigenic *Fusarium* species.

Keywords: PCR, tomato, *Fusarium,* mycotoxin gene clusters.

Introduction

Numerous species of the genus *Fusarium* are able to colonize a wide range of agricultural crops, including tomatoes. This is one of the most economically important genera due to the high pathogenic activity of its representatives. The phytotoxic effect of *Fusarium* leads to suppression of seed germination and plant growth, causing massive tissue necrosis, chlorosis, wilting, stem and root rot. [2]. Ultimately, there is a high risk of crop loss in tomato production, up to 80% depending on the region. [6]. Some *Fusarium* species, before colonizing tissues, produce a wide range of toxigenic compounds - mycotoxins, which weaken plants and cause suppression of immune responses [7]. In addition, mycotoxins contaminate food and feed products, reducing their quality, and have a negative impact on humans and livestock health [3]. *Fusarium spp.* can potentially contaminate tomato products with certain mycotoxins, which has attracted considerable interest due to the increasing frequency of their detection [5]. The most dangerous among them are fumonisins (FUM), deoxynivalenol (DON), zearalenone (ZEN) [1, 6]. Successful tomato production largely depends on the timely and exact identification of toxigenic species which represent a risk of potential mycotoxin accumulation in the final product. Information on toxigenic *Fusarium* species affecting tomato is needed for the development of better disease management strategies, including plant breeding.

The aim of the study was to reveal toxigenic *Fusarium* species – producers of mycotoxins that infect tomato plants at different phases of development. Molecular analysis was performed using nested-PCR assay and a set of species-specific primers for identification of *Fusarium spp.* and associated sequences from the Fumonisin biosynthetic (FUM) gene cluster, Trichothecene biosynthetic (TRI) gene cluster and Polyketide synthase (PKS13) genes.

Materials and methods

In the study plants of three tomato varieties – ′Cerasus′, ′Elvira′ and ′Deșteptarea′ (selection of IGFPP) were used. Throughout the vegetative season, mixed leaf samples from 12-15 asymptomatic plants of each variety were collected. Identification of *Fusarium* pathogens was carried out in seeding material, leaves collected at different stages of plant development (early growth, vegetative growth, flowering, fruits formation and growth) and in fruits at the stage of biological maturity. The leaves and fruits were thoroughly washed from soil before DNA extraction and grinded. Total DNA was extracted from 0,5 g of plant material according to the combination of

several protocols from ISO 21570(2005) (https://www.iso.org) and used at once for nested-PCR analysis.

A set of home-designed primers for *Fusarium* spp. detection was created based on species-specific regions of translation elongation factor 1-alpha (tef1) gene sequences of *F. verticillioides, F. oxysporum, F. pseudograminearum, F. solani, F. avenaceum, F. culmorum,* beta-tubulin (tub2) gene sequences of *F. equiseti, F. sporotrichioides,* oxygenase (fum6) gene sequences of *F. proliferatum,* and RNA polymerase II subunit (RPB2) gene of *F. nivale* from NCBI nucleotide collection presented in GenBank database using primer designing tool. Identification of the genes associated with the synthesis of fumanisin and zearalenone carried out using species-specific primers to sequences of the fumonisin biosynthetic (FUM) gene cluster and polyketide synthase (PKS13) genes.

PCR was performed in a 25 µl 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, 100 µg/ml-1 BSA, 0,2 mM of each dNTPs, 1,2 U Taq DNA polymerase (Thermo Fisher Scientific), 5 pM of each primer and 10 ng of DNA.

The first round of nested-PCR included 1 cycle at 95^0C for 3 min, 60^0C – 40 sec, 72^0C – 40 sec followed by 29 cycles: 95^0C – 40 sec, 60^0C – 40 sec, 72^0C – 40 sec, final elongation - 7 min. The conditions of the second round were: 95^0C – 40 sec, 60^0C – 40 sec, 72^0C – 40 sec, final elongation - 7 min (30 cycles). Amplification was carried out using MiniAmp™ Thermal Cycler, 96 wells, Thermo Fisher Scientific.

The products of amplification were separated in 1,5% agarose gel in the presence of 100 bp DNA ladder (Thermo Fisher Scientific) at the 6V/cm in TBE migration buffer (pH 8.0) with ethidium bromide, viewed in the UV (302 nm) and photographed.

Results and discussions

Development of fusarium infection of tomato plants was monitored throughout ontogenesis using primers complementary to nonspecific regions of the TEF1 genes of *Fusarium spp*. Mixed leaf samples were collected from the same plants of each variety at two-week intervals. Mass infection of tomato genotypes was observed in the middle of the vegetative season, at the flowering phase. This samples have been selected for subsequent tests using species-specific primers to *Fusarium spp.* Finally, seeds, leaves at the flowering phase, fruits at the stage of biological maturity were studied. The results of DNA analysis of tomato genotypes at some phases of development with the primers to *F. oxysporum, F. verticillioides, F. avenaceum, F. sporotrichioides, F. solani, F. equiseti, F. nivale, F. pseudograminearum, F. culmorum, F. proliferatum* are presented in Table 1.

Table 1

The identification of *Fusarium* species in tomato varieties during ontogenesis

Pathogens \ Genotype	Sowing material			Leaves (flowering phase)			Fruits		
	C	E	D	C	E	D	C	E	D
F. oxysporum	-	-	-	-	+	+	-	-	-
F. verticillioides	-	+	+	-	-	-	-	-	-
F. avenaceum	-	-	-	-	+	+	+	+	-
F. sporotrichioides	-	-	-	-	-	-	-	-	-
F. solani	-	-	-	-	-	+	-	-	-
F. equiseti	-	-	-	-	+	+	-	-	-
F. nivale	-	-	-	-	-	-	-	-	-
F. pseudograminearum	-	-	-	-	-	-	-	-	-
F. culmorum	-	-	-	-	-	-	-	-	-
F. proliferatum	-	+	+	-	-	-	-	-	-

C - ′Cerasus′, E - ′Elvira′, D - ′Deșteptarea′

In the sowing material *F. verticillioides, F. proliferatum* were determined in ′Elvira′ and ′Deșteptarea′ varieties. At the flowering phase in the leaves of the same varieties were identified *F. oxysporum, F. avenaceum, F. equiseti*. In addition, in ′Deșteptarea′ variety was revealed *F. solani.* In the fruits of ′Cerasus′ and ′Elvira′ varieties only *F. avenaceum* was identificated. Specific fragments corresponding to certain species of *Fusarium* are presented on Figure 1.

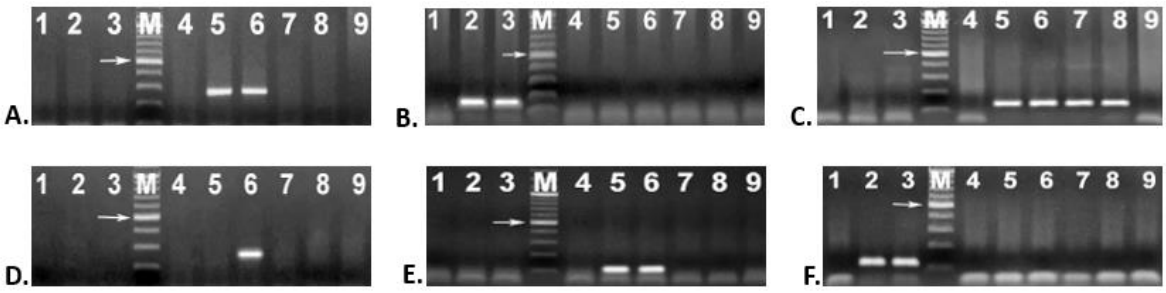


Figure 1. Electropherograms of *nested*-PCR products amplified on the DNA isolated from the tomato plant material using primers to *Fusarium* species. A. *F. oxysporum,* B. *F. verticillioides,* C. *F. avenaceum,* D. *F. solani,* E. *F. equiseti,* F. *F. proliferatum.* 1, 4, 7: ’Cerasus’, 2, 5, 8: ’Elvira’, 3, 6, 9: ’Deșteptarea’; 1-3: seeds, 4-6: leaves, 7-9: fruits. M - 100 kb DNA ladder (arrow indicates the 500 bp fragment).

The positive signals in the second round of *nested*-PCR are marked with the presence of following amplicons on electrophoresis: *F. oxysporum* – 290 bp length, *F. verticillioides* – 140 bp, *F. avenaceum* – 160 bp, *F. solani* 288 bp, *F. equiseti* – 104 bp, *F. proliferatum* – 135 bp. All fragments resulting from *nested*-PCR correspond to the parameters calculated using the BLAST software.

Molecular analysis showed that most cases of tomato plants infection occurred during the flowering phase. At this stage observed a significant increase in the detection of *Fusarium* species. Tomato fruits were less infected compared to sowing material (seeds from the 2015 harvest). At the same time, ′Cerasus′ showed the lowest susceptibility to *Fusarium spp.*, since the listed in this study *Fusarium* species in seeds and in leaves at the flowering phase were not identified at this variety.

Tomato samples infected with toxigenic *Fusarium* species were studied using specific primers for the identification of genes associated with mycotoxin synthesis. The results of molecular analyses summarized in the table below:

Table 2

Identification of gene sequences involved in the synthesis of mycotoxins in tomato plants infected by pathogens from genus *Fusarium*

Mycotoxin gene clusters / Genotype		FUM gene cluster *(F. proliferatum)*	FUM gene cluster *(F. verticillioides)*	Polyketide synthase (PKS13) genes *(F. equiseti)*
Seeds	**Elvira**	+	+	
	Deșteptarea	+	+	
Leaves	**Elvira**			+
	Deșteptarea			+

It was found that *F. proliferatum* and *F. verticillioides,* identified in the seeds of the tomato varieties - 'Elvira' and 'Deșteptarea', contain the sequences of FUM gene cluster involved in the synthesis of Fumonisin B1. *F. equiseti* detected in the leaves of these varieties contains Polyketide

synthase (PKS13) gene sequences involved in the synthesis of Zearalenone. *Nested*-PCR results can be visualized on the figure 2.

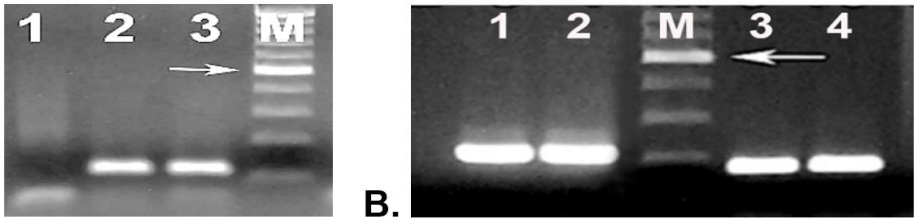


Figure 2. Nested-PCR products amplified using primers to sequences associated with mycotoxin synthesis and DNA from samples with defined toxigenic *Fusarium* species. A. Fum genes cluster sequensis of *F. proliferatum* (seeds). 1. Negative control, 2. 'Elvira', 3. 'Deșteptarea', B. 1, 2. Fum gene cluster sequensis of *F. verticillioides* (seeds); Polyketide synthase (PKS13) genes sequensis of *F. equiseti* (leaves). 1, 3.'Elvira', 2, 4. 'Deșteptarea'. M - 100 kb DNA ladder (arrow - 500 bp fragment).

Molecular analysis demonstrated that seeds collected in 2015 and intended for planting had a higher presence of *Fusarium* species with associated mycotoxin-producing sequences. This situation probably depends on the duration and conditions of seed storage. It is known that pathogenic fungi of seeds can serve as a source of disease development in plant ontogenesis [4]. Therefore, it is necessary to control for phytosanitary quality of long-term storage seeds. In addition, *Fusarium* sequences associated with the synthesis of mycotoxins have not been identified in tomato fruits.

Nowadays, is observed increasing of mycotoxin contamination in tomatoes and derived tomato products at all stages including cultivation, harvesting, production and storage [6]. Integrated management of pathogenic fungi and their mycotoxins is essential to prevent crop losses and human health issues. The search for tomato varieties that manifest low susceptibility to certain species and races of *Fusarium* is one of the beneficial ways of controlling this important plant pathogens and represents an effective preventive measure to reduce the contamination of agricultural products with mycotoxins. This is an environmentally favorable approach that reduces the risk of plant diseases and contamination of food chains. In this study, ′Cerasus′ showed the lowest susceptibility to *Fusarium* pathogens and is thus a promising candidate for future studies assessing resistance to *Fusarium* species.

Conclusions

Of the *Fusarium* species listed in the study, *F. proliferatum, F. oxysporum, F. verticillioides, F. avenaceum, F. equiseti, and F. solani* were identified at some phases of tomato development.

Molecular analysis showed that at the flowering phase there was a significant increase in fusarium infection of some tomato genotypes.

F. proliferatum and *F. verticillioides,* identified in the seeds of 'Elvira' and 'Deșteptarea', contain the sequences of FUM gene cluster involved in the synthesis of Fumonisin B1. *F. equiseti* detected in the leaves of these varieties contains Polyketide synthase (PKS13) gene sequences involved in the synthesis of Zearalenone.

The *Fusarium* species considered in the work with associated mycotoxin-producing sequences were not identified in the fruits at the stage of biological maturity.

Toxigenic *Fusarium* species and associated mycotoxin-producing sequences were not identified in the seeds and leaves of ′Cerasus′ variety. ′Cerasus′ showed the lowest susceptibility to *Fusarium* pathogens and is thus a promising candidate for future studies assessing resistance to specific *Fusarium* species.

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