

# MOLECULAR DETECTION OF FUSARIUM INFECTIONS IN WHEAT - A MEASURE OF QUALITY ASSESSMENT

## MOLEKULARNA DETEKCIJA FUSARIJUMSKIH INFEKCIJA U PŠENICI – MERE PROCENE KVALITETA

Sorina POPESCU, Oana-Maria BOLDURA, Aurica BOROZAN, Emilian MADOSA  
Banat University of Agricultural Sciences and Veterinary Medicine  
"King Michael I from Romania" Timisoara, Romania  
e-mail: sorinapopescu@usab-tm.ro

### ABSTRACT

This paper aimed to evaluate 50 wheat samples collected from different western Romanian locations based on microbiological, molecular, and toxicogenic assays to determine their correlation when species of the genus *Fusarium* were analyzed. The presence of toxins determined by biochemical ELISA (Enzyme-Linked Immunosorbent Assay), the DNA analysis based on PCR (Polymerase Chain Reaction), and even accurate species identification using specific gene sequencing were used to evaluate the fungal early infection. Considering that in Romania the prevalence of *Fusarium graminearum*, and *Fusarium proliferatum* infections is the most important, it can be stated that the screening with primers specific to fungal species ensures a preliminary test for fungal infection identification before performing the test for mycotoxins.

**Key words:** wheat, *Fusarium*, PCR analysis, barcoding.

### REZIME

Ovaj rad imao je za cilj da proceni 50 uzoraka pšenice prikupljenih sa različitih lokacija u zapadnoj Rumuniji, na osnovu mikrobioloških, molekularnih i toksikogenih testova, kako bi se utvrdila njihova korelacija kada su analizirane vrste roda *Fusarium*. Za procenu rane gljivične infekcije korišćeno je prisustvo toksina utvrđeno biohemijskom ELISA testom (Enzime-Linked Immunosorbent Assai), analizom DNK na osnovu PCR (Polimerase Chain Reaction), pa čak i tačna identifikacija vrsta korišćenjem specifičnog sekvenciranja gena. S obzirom da je u Rumuniji rasprostranjenost infekcija *Fusarium graminearum* i *Fusarium proliferatum* najvažnija, može se konstatovati da proveravanje prajmerima specifičnim za gljivične vrste obezbeđuje preliminarni test za identifikaciju gljivične infekcije, pre nego što se izvrši test na mikotoksine.

**Ključne reči:** pšenica, *Fusarium*, PCR analiza, bar kodiranje.

### INTRODUCTION

The genus *Fusarium* is considered the most important group of pathogenic fungi due to its diversity, widespread and especially their involvement in many plants diseases. Their importance is primarily related to the phytopatogenicity of certain species that cause large losses in the production of major crops, but also the contamination with their toxins, reducing the market value. The presence of toxins makes impossible the introduction of contaminated grains in the food chain both for humans and animals. The most important classes of mycotoxins are trichothecenes, fumonisins, and zearalenone. The presence of toxins is usually determined by biochemical ELISA (*Enzyme-Linked Immunosorbent Assay*) and HPLC (*High Performance Liquid Chromatography*) methods. But the progress of DNA analysis set up to the development of PCR (Polymerase Chain Reaction)-based methods to screen the fungal early infection, chemotype identification and even accurate species identification using specific gene sequencing (*Bozac et al., 2016*).

Therefore specific primers for different fungal species have been developed overtime:

*F. graminearum* and *F. culmorum*, *F. poae*, *F. cerealis*, *F. avenaceum* and *F. verticiloides* (*O'Donnell et al., 2004*).

Other sets of primers targeting different genes belonging to the TRI cluster could be used to identify NIV, 15-ADON and 3-ADON chemotypes within the *F. graminearum* species (*Alexander et al., 2011*). This cluster is involved in the biosynthesis of trichothecens produced by different species of the genus *Fusarium*. Primers for the Tri13 and Tri 7 genes were recommended by *Jurado et al.* in 2006 and those for the Tri 3

gene were designed by *Ward et al., in 2001*. Identification of several specific regions such as internal transcribes spacer (ITS) sequences, IGS (intergenic spacer) (*Yli-Mattila, 2004*), the gene encoding  $\beta$ -tubulin (*Yli-Mattila, 2004*) and the gene encoding 1- $\alpha$  translation elongation factor (*Geiser et al., 2004*) marked the transition to more specific identification of *Fusarium* species. The sequencing of these regions, followed by the construction of high-specific PCR primers, allowed the development of rapid and accurate identification, differentiation of toxicogenic and non-toxicogenic species, and investigation of mycotoxin biosynthesis pathways. Also, obtaining information about these sequences allowed not only the qualitative determination of a certain species, but also the quantitative determination of the fungal biomass.

The aim of this paper was to evaluate 50 wheat samples collected from different western Romanian locations based on microbiological, molecular and toxicogenic point of view.

### MATERIAL AND METHODS

The DNA was extracted from fungal cultures with the Norgen's Fungi/Yeast Genomic DNA Isolation Kit. For PCR amplifications the following composition was used: Green master mix Promega-12.5 $\mu$ l, primer 1 - 1 $\mu$ l, primer 2 - 1 $\mu$ l and sterile distilled water till 25  $\mu$ l. The amplification program following with the literature data, the annealing temperature being correlated with the used primers (Table 1). The amplified products were separated by 1.8% agarose gel electrophoresis and visualized by UV light in ethidium bromide presence. For sequencing the gel fragments were excised from gel and the DNA was purified with Monarch DNA Gel Extraction Kit

(BioLabs New England), following the manufacturer's instructions. The DNA fragments purified from gel were sent for sequencing to Macrogen, Netherlands. The Fusarium strains were isolated on PDA (Potato Dextrose Agar) medium and the unisporal isolates were selected. They were further developed for 21 days on autoclaved wheat medium, and the extraction was performed from 5 g of mycelium. The mycotoxins from the extracts were determined with the ELISA kits R-Biopharm Fumonisin and R-BiopharmDON, following the manufacturer's instructions.

Table 1. The primers sequences and the amplification conditions

Specific gene	Primers sequences	Amplification conditions	Amplicon size
<i>F. graminearum</i>	5' GTTGATGGGT AAAAGTGTG 3' 3' CTCTCATATA CCCTCCG 5'	25 cycles 53°C/ 30sec	500
<i>F. culmorum</i>	5' ATGGTGAACCTCGT CGTGGC3' 5' CCTTCTTACGCC AATCTCG 3'	25 cycles, 65°C/ 30sec	450
<i>F. proliferatum</i>	5' CGGCCACCAGA GGATGTG 3' 3' CAACACGAATCG CTTCCTGAC 5'	25 cycles, 65°C/ 30sec	230
<i>F. verticillioides</i>	5' CGCACGTATAGA TGGACAAG 3' 3' CACCCGCAGAA TCCATCCATCA5'	25 cycles, 65°C/ 30sec	700
<i>1-α translation elongation factor (TEF)</i>	5' ATGGGTAAGGA GGACAAGAC 3' 3' GGAAGTACCAG TGATCATGTT 5'	35 cycles, 53°C/ 30sec	650

## RESULTS AND DISCUSSIONS

In the first stage of research the collected wheat seeds were inoculated on specific media and morphological analyzed on macroscopic and microscopically level to identify the genus. 50 samples, considered to belong to the genus *Fusarium* were further analyzed. Take into account that not all strains produce toxins and there are wide variations in its amount, the study of the toxicogenic potential was an important step in assessing the toxicological risk. Therefore, the extracts obtained from 50 unisporal isolates were subsequently subjected to deoxynivalenol (DON) detection and quantification based on ELISA technique (Fig. 1).

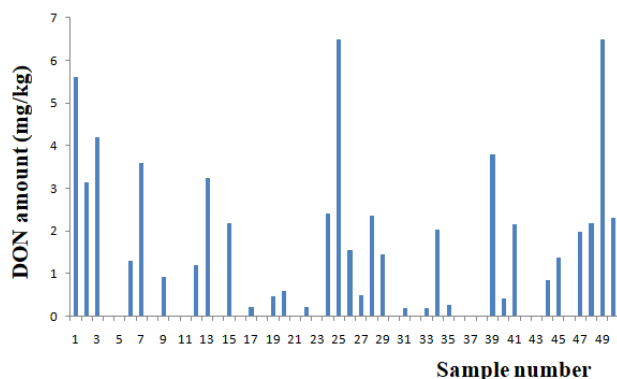


Fig. 1. The content of deoxynivalenol (DON) for the analyzed samples

The DON analysis was performed because this is the most widespread and at the same time the best monitored, being also an important factor of the pathogenicity and aggressiveness of this genus (Mesterhazy, 2002).

It was pointed out that 32 samples produced DON, with values between 0.2 mg/kg and 6.5 mg/kg, with amplitude of variation of 6.30 and a very high variability. Further on they were the subject of DNA analysis to establish the correlation between DON detection and PCR identification of genes specific to a specific *Fusarium* species.

For all of the positive samples for DON toxin the DNA was extracted and amplified with the primers specific for *Fusarium graminearum* specie. The amplification products were analyzed by agarose gel electrophoresis and visualized in ethidium bromide and UV presence (Fig. 2). All the samples were positive, pointing out the correlation between the PCR test based on *Fusarium graminearum* primers and the DON detection.

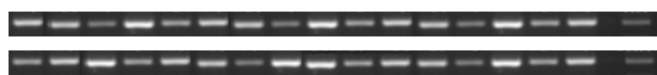


Fig. 2. The agarose gel electrophoresis of the products amplified with *Fusarium graminearum* specific primers

To confirm the identity of this fungal species several samples (10) were subjected to the barcoding procedure namely the sequencing of the translation elongation factor 1-α gene (TEF) which is known as a taxonomic marker for fungi. Therefore the DNA was amplified with the primers specific for the TEF gene (Fig. 3).

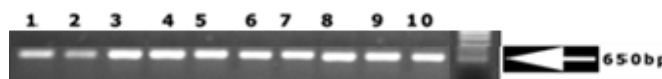


Fig. 3. The agarose gel electrophoresis of the products amplified with TEF (1-α translation elongation factor gene) specific primers

The positive bands were excised from the gel and the DNA was purified. Each purified DNA sample was sequenced and as an example, the sequence of one of the analyzed samples is presented.

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CCCCATTCCGGTGACGGTAGCGTCTGATAGCCATGTTAGTATGAGA
ATGTGATGACAGCATTGGTGACAACATACCAATGACGGTGACATA
GTAGCGAGGAGTCTCGAAGCTCCAGAGGGCGATATCAATGGTGAT
ACCACGCTCAGCTCGGCTTTGAGCTTGTAAGAACCAGGGCGTA
CTTGAAGGAACCCCTTACCGAGCTCGGCGGCTTCTATTGACAGGT
GGTTAGTGACTGTTTGACACGTGATGATGACCGCCAGGGGAATGG
ATTGTGGGAAGAGGGTCGACCCCTCGTCTATCGACTGGCGGGAT
TACTTTGTCAACCCGGGAAATAATACCATCTCATATATTAGTTAAAT
CTTCACTATATCCGGAGCCCAATCCACGGTCTATTCATTCC
AGAGCCACCATTATTACTGGTATAACTATCAAAAAATTATTATGA
CGCATGAACTGTTACAATTACTTATAACATGGTCTCTGTTAGGTCC
TGACGTCCTAATCTCGCGATTAATATTGATATCCTTACCTACCA
TTCCATCATACCAATAGTAGATATACGTACCATTACAGTTATATC
CGCCCTTATTGTTGCC
    
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Since 2004 a database has been set up containing thousands of sequences of genes specific to the genus *Fusarium*, which can be easily accessed through the *Fusarium* ID site. Besides, another much larger database NCBI (National Centre for Biotechnology Information), is usable by accessing the BLAST Nucleotide option. All sequences determined in the present experiment were compared with the databases and it was found that all belonged to the specie *Fusarium graminearum*. For example, the result of the comparison with the NCBI database is presented. Thus, an obvious correlation was established between the presence of DON toxin and the PCR test used to identify a species-specific gene.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
<input type="checkbox"/> <a href="#">Fusarium graminearum strain 433-2 translation elongation factor 1-alpha (TEF1) gene .complete cds</a>	<a href="#">Fusarium grami...</a>	497	497	50%	5e-136	96.10%	2106	<a href="#">MH572248.1</a>
<input type="checkbox"/> <a href="#">Fusarium graminearum isolate 1772 translation elongation factor 1-alpha (TEF1) gene .partial cds</a>	<a href="#">Fusarium grami...</a>	497	497	50%	5e-136	96.10%	608	<a href="#">MF974407.1</a>
<input type="checkbox"/> <a href="#">Fusarium graminearum strain 16FQ4-15 translation elongation factor-1 alpha gene .partial cds</a>	<a href="#">Fusarium grami...</a>	497	497	50%	5e-136	96.10%	644	<a href="#">KY471842.1</a>
<input type="checkbox"/> <a href="#">Fusarium graminearum isolate A3 elongation factor 1a (tef1) gene .partial cds</a>	<a href="#">Fusarium grami...</a>	497	497	50%	5e-136	96.10%	672	<a href="#">KX269081.1</a>
<input type="checkbox"/> <a href="#">Fusarium graminearum strain 14XY2X-18 translation elongation factor 1-alpha gene .partial cds</a>	<a href="#">Fusarium grami...</a>	497	497	50%	5e-136	96.10%	644	<a href="#">KP247739.1</a>
<input type="checkbox"/> <a href="#">Fusarium graminearum isolate NE1c-16Fg translation elongation factor 1-alpha gene .partial cds</a>	<a href="#">Fusarium grami...</a>	497	497	50%	5e-136	96.10%	660	<a href="#">MT299367.1</a>

For the other 18 samples the amount of fumonisin was determined and positive results were obtained for nine of them, with values between 0.74 mg/kg and 5.03mg/kg,

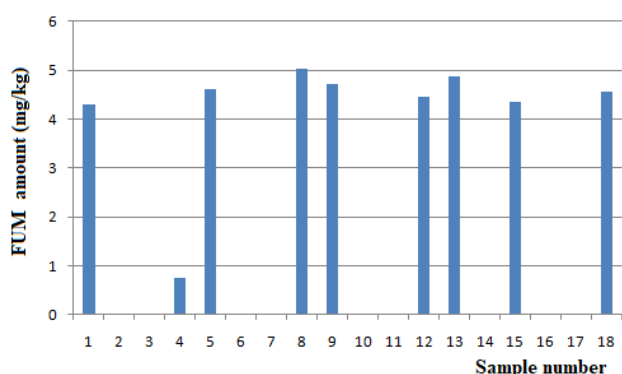


Fig. 4. The content of fumonisin (FUM) for the analyzed samples

Of these, five samples were *Fusarium proliferatum*, confirmed both by PCR and sequencing. The others were negative for PCR tests but were confirmed by sequencing as *Fusarium verticilloides*, *Fusarium andyiaze*, *Fusarium solani* and *Fusarium subglutinans* (Table 2). Another nine samples were negative in terms of both mycotoxins and molecular analysis.

Table 2. The correlation between the biochemical and molecular assays

Nr.	Sample code	FUM mg/kg (mg/kg)	Specie identified by specific PCR PCR	Specie identified by TEF sequencing
1	1	4,30ab		<i>F. verticilloides</i>
2	4	0,74c	<i>F. proliferatum</i>	<i>F. proliferatum</i>
3	5	4,60ab		<i>F. andyiaze</i>
4	8	5,03a	<i>F. proliferatum</i>	<i>F. proliferatum</i>
5	9	4,72ab		<i>F. solani</i>
6	12	4,45ab	<i>F. proliferatum</i>	<i>F. proliferatum</i>
7	13	4,35ab		<i>F. subglutinans</i>
8	15	4,86ab	<i>F. proliferatum</i>	<i>F. proliferatum</i>
9	18	4,55ab	<i>F. proliferatum</i>	<i>F. proliferatum</i>

## CONCLUSIONS

Of the 50 analysed samples, 32 were *Fusarium graminearum* and 5 *Fusarium proliferatum*, confirmed by morphological,

microbiological, immunological and molecular methods. Nine samples were negative for all tests performed and the others belonged to *Fusarium verticilloides*, *Fusarium andyiaze*, *Fusarium solani* and *Fusarium subglutinans*, confirmed only by sequencing. Considering that in Romania the prevalence of *Fusarium graminearum* and *Fusarium proliferatum* infections is the most important, it can be stated that the screening with primers specific to fungal species ensures a preliminary test for fungal infection identification before performing the test for mycotoxins.

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