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# Intra-strains diversity of expression of polymorphic *PKS4* gene in comparison in zearalenone production by *Fusarium graminearum* during *in vitro* cultivation*

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**Filamentous fungi belonging to the *Fusarium* genus are responsible for large economic losses due to their high pathogenicity and toxigenicity. *Fusarium sp.* may produce variety of mycotoxins, one of them is zearalenone (ZEA). The presence of the *PKS4* gene shows the possibility of zearalenone biosynthesis by *Fusarium sp.* In this study, in four *Fusarium graminearum* and one *Fusarium poae* strains the presence of *PKS4* genes and ZEA concentrations were determined. The presence of the *PKS4* gene was confirmed by classical polymerase chain reaction (PCR) in three of four strains of *F. graminearum*. One strain with no *PKS4* gene detected was found while still producing ZEA. In the present study, a real-time PCR assay has been successfully performed for the relative expression of *Fusarium* strains based on new designed primers targeting the *PKS4* gene involved in ZEA biosynthesis. Result shows that P56/4 strain of *F. graminearum* has the highest mRNA level, in the range of 12, what correlates to the high production of this mycotoxin. In this study, a real-time PCR assay has been successfully developed for the prediction of the production of ZEA by *F. graminearum* strains by PCR real-time techniques based on primers targeting the gene, *PKS4,* involved in ZEA biosynthesis. The special significance was pointed to occurring genes polymorphism.**

**Key words**: *Fusarium sp. PKS4*, ZEA, gene expression, polymorphism

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## INTRODUCTION

The most common pathogens in agriculture are fungi of the genus *Fusarium* (Mule & Logrieco, 1997; Leslie & Summerell, 2006). They are the cause of large economic losses due to their high pathogenicity and toxigenicity (O'Neil, 1986). In north-western and eastern European countries, the most common pathogenic *Fusarium* species are *F. graminearum* and *F. culmorum* depending on the climate of the country (Stenglein, 2009). *F. graminearum* is the most important *Fusarium* species in Central Europe and in large areas in North America and Asia (Yli-Mattila, 2010). Of 144 barley seed samples coming from 12 administrative regions of Quebec in 2000, 2001 and 2002, *F. graminearum* was the most common, and accounted for approximately 40% of the infected seed lots, but *F. poe* was the second most abundant species in 2000 and 2002 (Stenglein, 2009).

The *Fusarium* genus currently includes over 80 species. This number is increasing as studies based upon deoxyribonucleic acid (DNA) sequence comparison become more common (Cuomo *et al.*, 2007; Liu *et al.*, 2013). The *Fusarium graminearum* species complex was previously classified as a single species. Advanced phylogenetic analyses based on GCPSR sequences revealed the complex structure of this group of plant pathogens and the presence of 16 phylogenetic species (Gardiner *et al.*, 2014).

One of the most widespread toxin produced by *Fusarium spp.* is zearalenone (ZEA). It is classified into the group of non-steroid estrogens or mycoestrogens. The reduced form of zearalenone, zearalenol, has increased estrogenic activity (Edwards *et al.*, 2002; Bennett *et al.*, 2003). *F. poae* is able to synthesise fusarenon X, as well as trichothecenes, but no ZEA (Leslie *et al.*, 2006).

The genetics and biosynthesis of different fusarins have been the subject of intensive study (Brown, 2004), but the genetics and biosynthesis of ZEA have received comparatively little attention. Ward *et al.* (2002) confirmed that a *PKS* genes complex is responsible for biosynthesis of family related enzymes called polyketide (2002), which is responsible for ZEA activity. A search of genomic sequence of *Fusarium verticillioides, F. graminearum, F. oxysporum* and *F. solani* identified a total of 58 *PKS* genes (Brown *et al.*, 2012).

In the EU, the maximum limit value was determined for ZEA as 100 g $kg^{-1}$ in unprocessed cereals (European Commission Regulation No 1126/2007, Yli-Mattila, 2010). In spite of the dangerous characteristics of different *Fusarium* species, the genetic and environmental regulation of their mycotoxin, specially ZEA production and phytopathogenicity, is still poorly characterised (Yli-Mattila, 2010).

The methods like high performance liquid chromatography (HPLC), gas chromatography (GC) or liquid chromatography with tandem mass spectrometry (LC-MS/MS) are usually used to detect zearalenone. Because these methods are laborious and time-consuming new, rapid and sensitive methods to detect zearalenone-producing *Fusarium* strains are needed to prevent food and feed contamination. Many possibilities are concerned with prognostic molecular methods (Suchorzyńska & Misiewicz, 2009; Suchorzyńska, Spera & Misiewicz, 2009).

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*Accession number in GenBank: LC038084

**Abbreviations**: AZEA, Zearalenone; PDA, potato dextrose agar; PDB potato dextrose bullion; PDA, potato-dextrose agar; HPLC, high performance liquid chromatography, ACN, acetonitryl, PCR, polymerase chain reaction; GC, gas chromatography; LC-MS/MS, liquid chromatography with tandem mass spectrometry; RSD, relative standard deviation

**Table 1. Sequence of primers used in DNA amplification**

| Primers | Sequence 5′-3′ | PCR reaction conditions | Reference |
|---|---|---|---|
| P58SL<br>P28SL | AGT ATT CTG GCG GGC ATG CCT GT<br>ACA AAT TAC AAC TCG GGC CCG AGA | 94°C – 5 min,<br>[94°C – 1 min, 68°C – 1 min, 72°C – 1 min] x35,<br>72°C – 10 min | Hue *et al.* (1999) |
| PKS4R<br>PKS4F | GTG GGC TTC GCT AGA CCG TGA GTT<br>AGA TGG CCA TGG TGC TTC GTG AT | 94°C – 2 min,<br>[94°C – 30s, 59°C – 30s, 72°C – 1min] x35,<br>72°C – 11 min | Lysoe *et al.* (2006) |
| Fc01F<br>Fc01R | ATG GTG AAA CTC GTC GTG GC<br>CCC TTC TTA CGC AAT CTC G | 94°C – 5 min,<br>[94°C – 45 s, 66°C – 1 min, 72°C – 1 min] x5,<br>[94°C – 45 s, 64°C – 1 min, 72°C – 1 min] x5<br>[94°C – 45 s, 62°C – 1 min, 72°C – 1 min] x25,<br>72°C – 5 min | Nicholson *et al.* (1998) |
| Fg16NF<br>Fg16NR | ACA GAT GAC AAG ATT CAG GCA CA<br>TTC TTT GAC ATC TGT TCA ACC CA | 94°C – 5 min,<br>[94°C – 4 s, 66°C – 1 min, 72°C – 1 min] x5,<br>[94°C – 45 s, 64°C – 1 min, 72°C – 1 min] x5,<br>[94°C – 5 s, 62°C – 1 min, 72°C – 1 min] x25,<br>72°C –5 min | Nicholson *et al.* (1998) |
| Fg82R<br>Fp82F | CAA GCA AAC AGG CTC TTC CAC C<br>TGT TCC ACC TCA GTG ACA GGT T | 94°C – 2 min,<br>[94°C – 1 min, 55°C – 1 min, 72°C – 2 min] x40,<br>72°C – 5 min | Parry & Nicholson (1996) |

PCR was conducted in a final volume of 25 µl containing: 2.5 µl of each primer (2 µM); 13 µl of $H_2O$; 2.5 µl dNTPs (each 2.5 µM); 2.5 µl of 10 x Run buffer; 1 U (1 µl) of Run polymerase; and 1 µl of template DNA. PCR products were detected by electrophoresis on 1.5% agarose gel in 0.5% TBE buffer with ethidium bromide.

The aim of this work is to predict pathogenity, through the means of producing ZEA in five *Fusarium* strains, isolated from wheat seeds, in comparison with real production of this mycotoxin during *in vitro* cultivations and assessment of efficacy of classical primers.

## MATERIALS AND METHODS

**Materials**. From many isolates from wheat seeds, five strains isolates were chosen as representative examples from five voivoidships of Poland: świętokrzyskie (sample P7/4), opolskie (samples P48/1 and 48/6), śląskie (sample P56/4) and wielkopolskie (sample P63/23). Their localisation was combined with essential wheat areas in Poland. The strains are deposited in the Culture Collection of Industrial Microorganisms, prof. Waclaw Dabrowski Institute of Agricultural and Food Biotechnology, Warsaw. All strains were grown in potato-dextrose agar medium (PDA) plates at 20°C.

**Filamentous fungi isolation**. Kernels were plated on solid potato-dextrose agar (PDA) medium with the addition of 50% citric acid solution. After a few days of incubation at room temperature, the fragments of single hyphae of strains were transferred onto new plates with PDA plates. This was repeated at least three times to assure their purity. For the isolates, single spore cultures were performed according to Leslie & Summerell (2006).

**DNA extraction**. The mycelium was cultured in PDB liquid medium prior to DNA extraction. DNA extraction from fungal mycelia was carried out as described previously by Liu *et al.* (2000). In all samples, DNA content was between 200–300 ng $l^{-1}$.

**Primers and DNA amplification.** *P58SL* and *P28SL* primers, designed by Hue *et al.* (1999), were used for the identification of filamentous fungi isolates. Zearalenone-producing isolates of *Fusarium* spp. were evaluated by using the primer pairs *PKS4R* and *PKS4F*, as described by Lysoe *et al.* (2006). The following primer pairs were used for species differentiation: Fc01F and Fc01R; Fg16NF and Fg16NR (Nicholson *et al.* 1998); and Fp82F and Fp82R (Parry & Nicholson, 1996) (Table 1). Primers were purchased from Genomed (Genomed S.A., Warsaw, Poland).

**Designing primers for real-time PCR reaction**. Based on the gene sequence of polyketide synthase *PKS4* (GenBank accession no DQ19316) new primers were designed to RT PCR reaction. Products of RT PCR reaction with PKS4 primers were sequenced (Genomed, Warsaw) and deposited (GenBank accession no. LC038084). The obtained *PKS4* sequences were also compared with all the sequences from Fusarium ID Database (Geiser *et al.*, 2004), so as to avoid polymorphic sites which gave false negative results in a classic reaction. Sequence specificity of the primers was checked by a blast program (http://www.ncbi.nlm.nih.gov/BLAST/, Altschul *et al.*, 1990). Primers are listed in Table 2. DNA sequences of the *EF1alfa* gene used as a housekeeping gene (Brunner *et al.*, 2009) were retrieved from GeneBank and aligned using Vector NTI (Lu & Moriyama, 2004).

**Table 2. Primers used in real-time PCR**

| Primer | Sequence 5′-3′ | Amplicon size (bp) | Reference |
|---|---|---|---|
| EF-G-fw | AGGTATTAAGCAGTACATTTTCTC | 181 | Brunner *et al.* (2009) |
| EF-G-rev | GGACTAGACTCAAAATTAGTATTTG | | |
| PKS4_RT_F | GCCTCAGATGGAAACTCGTT | 91 | This study |
| PKS4_RT_R | TGTTGGTGTCATCTTCTGTAGC | | |

**Table 3. Real-time PCR reaction profiles**

| Primer sets | Real-time PCR profile | Reference |
|---|---|---|
| PKS4_RT_F PKS4_RT_R | 95°C – 15 min, 94°C – 15 s, 57°C – 30 s, 72°C – 20 s x 40 cycle | this study |
| EF-G-fw EF-G-rev | 95°C – 15 min, 94°C – 15 s, 57°C – 20 s, 62.5°C – 20 s x 40 cycle | Brunner *et al.* (2009) |

The PKS4_RT_F primer is positioned at the bases 88 to 108, whereas PKS4_RT_R is positioned at the bases 157–179. This set of primers amplified a product of 91bp in *F. graminearum*. A series of 10-fold dilutions of stock solution were prepared as the templates for generating a standard curve through real-time PCR reactions. Three biological replicates were used.

**Specificity of the PCR real time reaction**. The specificity of PCR real time reaction was tested using *Fusarium poae* strain 63/23, which has no ability to produce ZEA.

**Real-time PCR optimisation**. Temperature profiles were preliminarily tested with genomic DNA by using gradient PCR (Mastercycler, Eppendorf, Hamburg, Germany) (Tables 3).

**RNA extraction and cDNA reverse transcription**. RNA was extracted using the commercially available kit, RNease Plant Mini Kit (Qiagen), and purified from any residual genomic DNA using the RNase-Free DNase Set (Qiagen). The quality and quantity of the extracted RNA was assessed with a NanoDrop Spectrophotometer (Thermo Scientific) and by electrophoresis in 1% agarose (Sigma). The RNA samples were conserved with liquid nitrogen (–196°C).

The extracted RNA was then subjected to reverse transcription (cDNA synthesis) using the QuantiTect Reverse Transcription Kit (Qiagen) with oligo (dT) priming. The purity and yield of cDNA were determined using a NanoDrop Spectrophotometer (Thermo Scientific). The cDNA were stored at –20°C.

**Real-time PCR reaction and quantitative analysis of the data**. Real-time PCR analysis was performed in Rotor Gene 6000 instrument (Corbett Life Science) using the double-stranded-DNA-specific fluorochrome SYBR Green (Qiagen). *PKS4* gene expression analysis was carried out with the QuantiTect SYBR Green PCR Kit (Qiagen), the Maxima SYBR Green qPCR Master Mix (2x) (Thermofisher Scientific). As a reference gene, the *EFG* gene was used (Brunner *et al.*, 2009). The results of real-time PCR were analysed according to 2-ΔΔ method (Livak, 2001). The expression level was described in terms of the cycle threshold value (Ct), which is the number of cycles required to reach a certain fluorescence value (threshold). The data, expressed as Ct, was imported into a Microsoft Excel data sheet for the next, statistical analysis.

**Cultures for mycotoxin analysis**. The wheat grains were sterilised, cooled and inoculated with spores obtained by growing isolates (Joffe & Palti, 1975) on PDA slants with 10 ml of peptone water. They were then incubated for up to two weeks at room temperature (19–25°C) under varied lighting conditions. Following the dying of the grains, the mycotoxin content was determined.

**Zearalenone (ZEA) content analysis**. ZEA was determined using a HPLC instrument (Knauer, Berlin, Germany) consisting of a gradient pump K 1001, a loop dispensing valve with volume 100 µl, a chromatographic column RP-C18 Nucreosil 5 µm 4.6×250 mm with porosity 100 Å (Machery-Nagel Düren, Germany) with analogous precolumn, a device for the degassing of liquid connected to the chromatographic system, a fluorimetric detector RF/-10AXL operated at λ=274/446 nm (Shimadzu, Vernon Hills, IL, USA) and a computer with Eurochrom HPLC Software 1.65 (Knauer, Berlin, Germany) and a Canon printer.

A set for immunoaffinity chromatography was also used, consisting of a tripod, a vessel, wires, a syringe (Vicam, Watertown, USA), laboratory mill WŻ-1 (CZSS Spolem, Poland), an ultrasonic bath, (Buechler, Dusseldorf, Germany), a laboratory shaker Promax 1020 (Heidolph, Schwabach, Germany), a vacuum dryer (Büchi, Postwach, Switzerland), a heating block (Pierce New Haven, USA), a Waring blender, microsyringes for HPLC (Hamilton, USA), reaction vessels (Pierce, New Haven, USA) and typical laboratory glass.

Methanol:ACN:water mixture (8:46:46) was used as the isocratic mobile phase with the flow rate of 1 mL $min^{-1}$. The calibration curve was determined using ZEA standards of the 3.75/18.75/37.50 ng $mL^{-1}$ concentrations (corresponding to concentrations of the analyte in the samples, 10, 50 and 100 µg $kg^{-1}$ respectively).

Fifty grammes of the sample was ground, mixed with 5 g of NaCl and extracted for two minutes in a homogeniser with 100 mL of 90:10 v/v ACN:water solution. The entire mixture was transferred into a centrifuge tube and centrifuged for 10 minutes at an acceleration of 10730×g. Next, 10 mL of the extract, dissolved in 40 mL of water, was treated with ultrasounds in an ultrasonic bath for two minutes, then filtered through a Microfibre Filter (Vicam, Watertown, MA, USA). ZearalaTest® Immunoafinity Columns (IAC) (Vicam, Milford, USA) were used.

In the case of zearalenone, it is very important to maintain the proportion between organic solvent and water. Zearalenone is not easily soluble in water, therefore, the extraction has to take place with a high proportion of methanol and/or acetonitrile in the extraction mixture. Since too large a proportion of organic solvent in the extract causes elution of the analyte from the IAC columns, the extract was diluted with water to a maximum, with a minimum quantity of organic solvent capable dissolving the analyte.

The dissolved and filtered extract was cleaned up on IAC column: 10 mL of the extract was passed through the column at the 1–2 droplets per second rate. ZEA was eluted with 3 mL of methanol at the 1 droplet per second rate, then evaporated in a stream of nitrogen. Residues dissolved in 2 mL of ACN:methanol:water mixture (46:8:46) were submitted to analyses.

Method recovery rate and precision were tested on some wheat samples spiked with ZEA to the 50 µg $kg^{-1}$ level. The samples were previously verified as not containing the toxin. The average recovery rate (n=3 samples) was 100.4%. No relative standard deviation (RSD) exceeded 5%. ZEA limit of quantification was 10 µg $kg^{-1}$.

## RESULTS

Four strains are identified as *F. graminearum* and one as *F. poae*. All isolates were checked for cultivation for subsequent mycotoxin assay based on the presence of

**Table 3. Molecular data from the evaluation of *Fusarium spp*. isolates selected for *in vitro* cultivation for mycotoxin analysis**

| No. | Isolate | Species | Presence of *PKS4* gene | Zearalenone μg kg$^{-1}$ |
|---|---|---|---|---|
| 1. | P63/23 | *F. poae* | – | – |
| 2. | P7/4 | *F. graminearum* | – | 37.70 |
| 3. | P48/1 | *F. graminearum* | + | 94.58 |
| 4. | P48/6 | *F. graminearum* | + | 90.06 |
| 5. | P56/4 | *F. graminearum* | + | 89.65 |
| Detection limit 10 μg kg $^{-1}$ | | | | |

absent, + present

genes involved in mycotoxin synthesis. In conventional PCR, all *F. graminearum* strains, except one, gave a 400 bp product. There was no PCR product in the P7/4 (*F. graminearum*) or in the P63/23 sample (*Fusarium poae* strain).

These samples were subjected to chromatography analysis (Table 3) and these isolates were selected for *PKS4* gene expression analysis. The content of ZEA was different in the samples. In the P48/1, P48/6 and P56/4 samples, the content of ZEA was similar in amount, from 89 650 to 94 580 μg kg$^{-1}$. In the P/7 sample, the content of ZEA was lower, and lacked mycotoxin in the P63/23 sample (Table 3).

*PKS4* gene expression was analysed with real time PCR in the following isolates: P48/1, P48/6, P56/4, due to high mycotoxin concentration, P7/4, due to zearalenone production despite the fact that the *PKS4* gene was not detected by conventional PCR, and P63/23, to compare result with a species without the ability to synthesise zearalenone (Fig. 1). The increased *PKS4* expression was found to be almost 10-fold in P56/4 and over 6-fold in the P48/6 cultivations.

## DISCUSSION

Biochemical and genetic studies of *Fusarium* sp. conducted by Proctor *et al.* (1999), Ward *et al.* (2002) and others led to the identification of the genes responsible for biosynthesis of mycotoxins. These methods allowed for the mechanisms of zearalenone biosynthesis and the significance of the *PKS* gene complex to be described. The studies revealed the relationship between *PKS4* expression and biosynthesis of zearalenone. *PKS4* encodes an enzyme that stimulates the expression of another gene, a *PKS13* – encoding protein involved in the final stage of zearalenone biosynthesis. This protein catalyses the carbon addition to the final intermediate compound. Studies performed by Lysoe *et al.* (2006) indicated that *PKS4* is necessary for zearalenone biosynthesis.

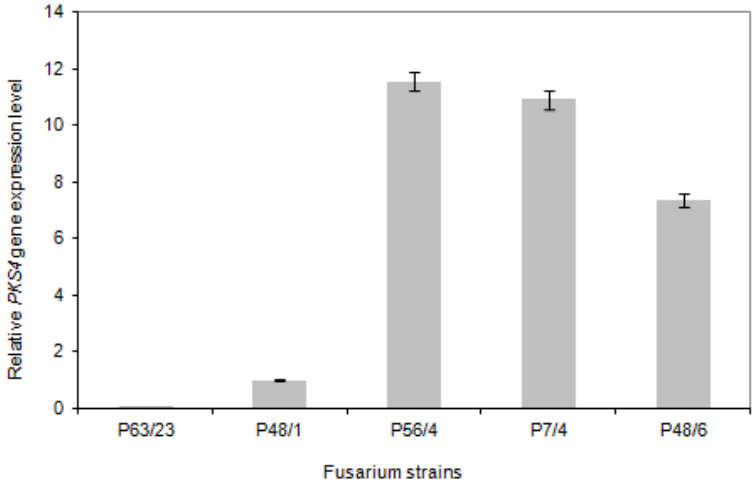


**Figure 1. Comparison of *PKS4* gene expression in five isolates of *Fusarium spp*.** Error bars indicate standard deviations.

The complete sequencing of the *F. graminearum* genome has allowed the identification of distinct regions of high diversity (Trail, 2009). Cuomo *et al.* (2007) have found that these regions are rich with infection-related genes, which may allow this fungus to adapt rapidly to changing environments or hosts. The availability of its genomic resources will allow the comparative analysis of *F. graminearum* and *F. poae*, for understanding the changing of gene clusters (Stenglein, 2009). Genes involved in the production of secondary metabolites and secreted proteins are enriched in non-conserved regions. They found evidence that non-conserved regions are generated by the sequestration of genes from multiple locations (Zhao *et al.*, 2014).

Research has shown zearalenone production in *Fusarium graminearum* strains in which the *PKS4* gene was not detected. As presented in studies performed by Lysoe *et al.* (2006), *PKS4* is necessary for zearalenone biosynthesis, which was proven by DNA fragment insertion into the *PKS4* gene resulting in stopped production of mycotoxin. However, the exact zearalenone synthesis gene mechanism is still not known. Gaffoor & Trail (2006) has proposed a possible ZEA production path in which the *PKS4* encoded enzyme catalyses the first 10 carbon additions, and after that reduced moiety is subsequently transferred to the *PKS13* encoded enzyme, where the remaining three rounds of C2 additions are catalysed. These results show the crucial role of the *PKS4* gene in ZEA synthesis.

With regard to the P7/4 isolate, no amplicon for the *PKS4* gene was detected in classical PCR reaction using PKS4F and PKS4R primers, although expression of this gene was observed using different, shorter PKS4_RT_F and PKS4_RT_R primers. The zearalenone concentration in this case was 37 700 μg kg$^{-1}$, relatively low, which confirm some structural changes in gene sequences, comparing to the rest *F. graminearum* strains. In the remaining isolates of *Fusarium graminearum*, mycotoxin content was approximately 90 000 μg kg$^{-1}$. Similar findings were observed previously by Stepien *et al.* (2012) in a few isolates of *Fusarium equiseti,* which indicates the possible polymorphisms of the *PKS4* gene and significant intra-specific diversity.

Some authors thought that the diversity and distribution of *PKS*s was attributed to gene duplication, divergence and differential gene loss rather than horizontal gene transfer (HGT) (Kroken *et al.*, 2003), but the analysis of Brown *et al.* (2012) suggested that HGT could be responsible for significant *PKS* diversity. It is also taken into consideration that the *PKS13* and *PKS4* genes functions together as a multiprotein complex. In addition, genes involved in the production of secondary metabolites and secreted proteins are enriched in non-

conserved regions, suggesting that these regions could also be important for adaptations to new environments, including adaptation to new hosts. Zhao *et al.* (2014) found evidence that non-conserved regions are generated by sequestration of genes from multiple locations. Gene relocations may lead to clustering of genes with similar expression patterns or similar biological functions, which was clearly exemplified by the *PKS2* gene cluster (Zhao *et al.*, 2014).

Baturo-Ciesniewska & Suchorzyńska (2011) confirmed that primers, recommended in the literature, for the *Fusarium* species were not completely effective. It was shown that from among the four primer pairs enabling the identification of *F. culmorum*, and therefore also to establish its presence in the material, only primers Fc01F/Fc01R seem to be fully effective in the case of Polish strains (Baturo-Ciesniewska & Suchorzyńska, 2011).

In our study, lack of the product of PCR was probably due its polymorphism or changes in genome. With regard to the P63/23 isolate (*F. poae*), molecular analysis indicated the absence of *PKS4* genes, and the expression analysis confirmed the lack of *PKS4* expression, which was reflected by no observed zearalenone production. The low content of this mycotoxin was detected in cultures inoculated with P7/4, isolate P48/1, and almost three times higher than in the P56/4 and P48/6 isolates. However, no correlation was found between the *PKS4* expression level and the quantity of zearalenone produced by the particular *Fusarium spp.* isolate.

A pathogen's toxicity is associated with high variations in virulence traits, the spread of isolates with specific attributes into new regions, the ability to produce three types of mycotoxins as well as fungicide resistance (Chen & Zhou, 2009; Yli-Mattila *et al.*, 2009; Yli-Mattila, 2010; Zhang *et al.*, 2012; Sploti *et al.*, 2014).

This observation is a challenge and warrants further studies in this subject, especially on the impact of genes polymorphisms on mycotoxin production and pathogenicity of filamentous fungi (Baldwin *et al.*, 2010).

Production of zearalenone was also observed when neither the *PKS4* nor the *PKS13* gene was detected. In our results, expression of *PKS4* gene is observed with specially designated, new, shorter starters, avoiding polymorphic sites. This observation is a challenge and warrants further studies in this subject, especially on the impact of polymorphisms on mycotoxin production and pathogenicity of filamentous fungi.

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