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ISOLATION AND MOLECULAR IDENTIFICATION OF FUNGAL ISOLATES FROM STORED CEREALS USING PCR-RFLP METHOD

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ABSTRACT

Contamination of grain cereals with toxic metabolites of fungi, both pathogenic and saprotrophic, is one of the particularly important problems in global agriculture. The aim of the current study was molecular identification of fungi isolates from different samples of stored cereals and evaluate the utility of PCR-RFLP of the ITS region technique. The results established that the most abundant species were found belonging to *Aspergillus* genera (50%), followed by *Fusarium* spp. (19%) and *Penicillium* spp. (19%). *Aspergillus flavus* was the most frequent species, representing almost 40% of the isolates belonging to the genus *Aspergillus*. Also were identified as *Aspergilus versicolor*, *Aspergilus ruber* and *Aspergilus niger* by molecular analysis representing 10% each.

Keywords: wheat, electrophoresis, fungal contamination, DNA amplification, DNA sequencing.

INTRODUCTION

Cereals are natural substrates for fungal development due to their nutritional composition rich in starch and protein, proving necessary carbohydrate and nitrogen for development of micromycetes thus contamination leading to financial and food safety losses (Fleurat-Lessard, 2017).

Food grains may be stored at different levels in the food supply chain in silos, warehouses, bags, containers, traditional storage structures, or in other defined units. The occurrence of molds in stored grains is influenced by a range of abiotic and biotic factors like temperature, water availability and intergranular gas composition, contaminant molds, insect pests, rodents and preservatives which are added to conserve moist grain for animal feed (Magan et al., 2003; Fleurat-Lessard, 2017) and represents an important problem in different countries worldwide.

Many species of fungi that are found on the surface or inside grains at harvest may cause spoilage in stored grains and produce mycotoxins that severely decrease crop value and are harmful to animal and human health that consume the contaminated feed or grain products (Placinta et al., 1999; Magan et al., 2010; Shiju et al., 2010; Gonzales Perreyra et al., 2011; Belkacem-Hanfi et al., 2013; Jedidi et al., 2018; Kumari et al., 2019).

Studies on the several filamentous fungi (*Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria*) which attacked stored cereal grain and are responsible for production of toxic metabolites for humans and animals (Belkacem-Hanfi et al., 2013; Comby et al., 2016; Gdanetz and Trail, 2017) are well documented.

These fungi can be identified by traditional plating techniques based on the morphological characteristics such as the shape and the size of the conidia, the presence/absence of chlamydospores and also the colony morphology. However conventional methods used for fungal detection are labour and time-consuming require mycological expertise and not always very specific (Jurado et al., 2006) In general, it is difficult to distinguish between species having similar

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morphological characteristics, particularly when using traditional methods.

Thus the molecular approaches have been developed for toxigenic fungi studies like polymerase chain reaction (PCR) techniques which are rapid, sensitive, specific and reliable diagnosis methods in species identification without additional confirmation steps (Nicholson et al., 1998; Jurado et al., 2006; Suanthie et al., 2009; Atoui et al., 2011; Datta et al., 2011; Kumari et al., 2016; Sadhasivam et al., 2017; Jayachandran et al., 2019).

However, a widely used method is PCR-RFLP a powerful technique generally used for microbial identification and classification even at species level, by which PCR amplification of a target region is performed, followed by digestion of the fragment amplified with restriction enzymes. Separation of the obtained fragments is performed by electrophoresis to visualize the differences between the restrictions profiles obtained.

This technique was used for the rapid identification of the large number of fungi (Somashekar et al., 2004; Dupont et al., 2006; Llorens et al., 2006; González-Salgado et al., 2009; Diguță et al., 2011; Kizis et al., 2014; Ahmad et al., 2014; Kachuei et al., 2015). No studies have been reported in Romania on the use of the PCR-RFLP method for identification of fungitoxic species from stored grains.

The studies of the Romanian authors were focused on the analysis of the cereals mycoflora (Tabuc et al., 2009; Cornea et al., 2013; Misca et al., 2014; Dudoiu et al., 2016; Stanciu et al., 2017) and their content of mycotoxins (Placinta et al.,1999; Alexa et al., 2013; Misca et al., 2014; Bozac et al., 2016; Gagiu et al., 2018).

In light of these informations, the objective of present study was to examine the fungal contamination of different stored wheat samples using molecular tests species specific RFLP-PCR assays.

MATERIAL AND METHODS

Two hundred samples of contaminated wheat were collected from different grain warehouse located in different areas of Păulești, Prahova County in the years 2015-2017. From each warehouse a sample of 1 kg was collected from the front, from the center and from different points in the horizontal depth of the grain mass and also in the area close to the walls of the warehouse.

The water content of cereal seeds varied between 10-14%. The collected samples were kept in sterile plastic bags during transport and 100 g of each sample was frozen in liquid nitrogen, lyophilized and ground in a fine puberty. The samples were kept at 4°C until analysis.

1. Fungal DNA extraction

The fungal isolates were grown in PDB (Potato Dextrose Broth, VWR Chemicals) liquid medium, at a temperature between 28-30°C, for 96 hours. The fungal biomass was treated with liquid nitrogen in order to break down the cell wall. Subsequently, the fungal suspension obtained was centrifuged at 10.000 rpm for 10 minutes, thus resulting a sediment of approximately 100-150 mg. The obtained sediment was used as a base for fungal DNA extraction using the ZR Fungal/Bacterial MiniPrep kit (Zymo Research, USA).

Following the protocol, 750 µl of lysis solution was added to the fungal sediment and the obtained mixture was vortexed at maximum speed for 5 minutes. The obtained suspension was centrifuged at 10000xg for 1 minute; The binding buffer (Fungal/ Bacterial DNA Binding Buffer) - supplemented with 0.05% beta-mercaptoethanol (v/v) was added to the filtered suspension; the resulting mixture was centrifuged at 10000xg for 1 minute. A pre-wash buffer (DNA Pre-Wash buffer) was added and a new centrifugation was performed. Next, wash buffer (Fungal/Bacterial DNA Wash Buffer) was added to the supernatant and again the mixture was centrifuged 10000xg for 1 minute. In the final step, the DNA was resuspended in 75 µl of DNA elution buffer.

2. DNA amplification and sequencing

RFLP-PCR-ITS specific assays were performed using ITS1 (5'-

TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') amplification primers, retained to amplify the 5.8S-ITS region.

PCR amplification reactions were performed in a final volume of 50 µl containing 10X DreamTaq Green Buffer (containing 20 mM $MgCl_2$), 100-150 ng/DNA, 10 mM dNTP, 10 µM from each primer and DNA polymerase 5 U/µL. The reaction mixture was vortexed and then subjected to PCR reaction.

PCR amplification was performed in a thermal cycler (Multi Gene, Labnet), the reaction mixture being subjected to several thermocycles with the following profile: 1 cycle representing the initial denaturation (3 minutes at 94°C), 35 cycles consisting of denaturation (1.5 minutes at 94°C), hybridization (1.5 minutes at 55.5°C) and elongation (2 minutes at 72°C), followed by 1 cycle for final elongation (10 minutes at 72°C) and then the final cycle at 40°C.

Regarding the digestion of PCR products of the fungal isolates obtained it was performed using the following restriction enzymes (Thermo Fisher Scientific):

*Cfr*9I:	*Hae*III:	*Hinf*I:	*Mse*I:	*Sdu*I:
5'...C^CCGGG..3' 3'...GGGCC^C..5'	5’..GG^CC..3’ 3’..CC^GG..5’	5’...G^ANTC...3’ 3’...CTNA^G...5’	5’...T^TAA...3’ 3’...AAT^T...5’	5’...GdGCh^C...3’ 3’...C^hCGdG...5’

The reaction mixture consists of PCR product, phosphate buffer, nuclease-free water and restriction enzymes. The enzymatic digestion was performed on a water bath during 4 hours at 37°C for *Sdu*I, *Hinf*I; *Hae*III; *Cfr*9I and at 65°C for the enzyme *Mse*I.

3. DNA electrophoresis

The quality of the isolated fungal DNA was verified using agarose gel electrophoresis. The samples were loaded into a 2% agarose gel prepared in 1X TBE buffer (Tris-Borate-EDTA) containing 10 mg/mL ethidium bromide. 8 µl of PCR product, respectively 10-15 µl of restriction product were charged and the electrophoresis was performed using 1X TBE buffer at 90 V, for 1 hour until the front of the blue bromophenol dye migrated near the end of the gel. The molecular standard used was DNA Ladder 100 bp (GeneRuler 100bp Plus DNA Ladder, Thermo Scientific, USA).

Subsequently, PCR and restriction products were visualized using a UV transilluminator (GelDoc-IT Imaging Systems) by exposing ethidium bromide to UV light (λ=250-310 nm). The dimensions of the PCR products and the restriction fragments obtained were estimated by comparison with a standard length of DNA (GeneRuler 100bp DNA Ladder, Thermo Fisher Scientific). The analysis of the obtained restriction profiles was performed by comparing with the restriction profiles (theoretically obtained using the program http://biotools.umassmed.edu/tacg4/) the sequences already existing in the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/nuccore/).

RESULTS AND DISCUSSION

The initially performed morphological analysis (macroscopic and microscopic) on the 16 studied fungal isolates showed that the mycotoxigenic microflora detected on stored cereals is dominated by three important genera: *Aspergillus, Fusarium, Penicillium* (Figure 1).

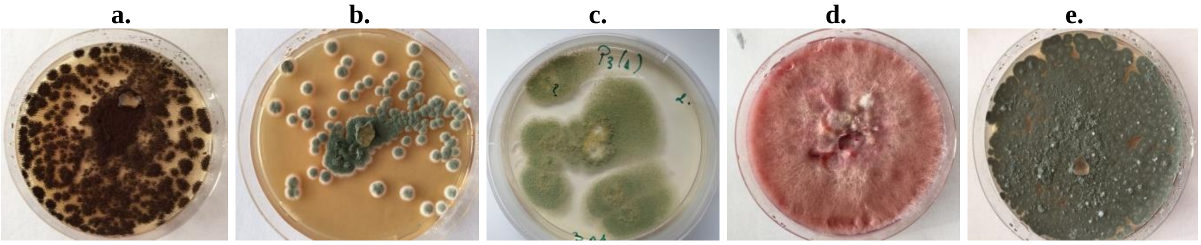

Figure 1. Isolated fungal strains on PDA media
a. *A. niger*; b. *A. parasiticus*; c. *A. flavus*; d. *Fusarium* sp.; e. *Penicillium* sp.

However, classical methods are not 100% efficient and at the same time they require high experience of researchers in terms of mold taxonomy. Thus, it is important that these mycotoxic molds to be identified quickly and accurately to ensure the safety of consumers and animals. Given that, an alternative to conventional microbiological techniques for diagnosing phytopathogenic fungi is represented by PCR molecular methods.

Efficient identification and discrimination of fungal species was performed with the PCR-RFLP method by selecting the 5,8S-ITS region. Following the amplification using the universal primers ITS1/ITS4 there were obtained PCR products with dimensions of approximately 540-600 bp (Figure 2).

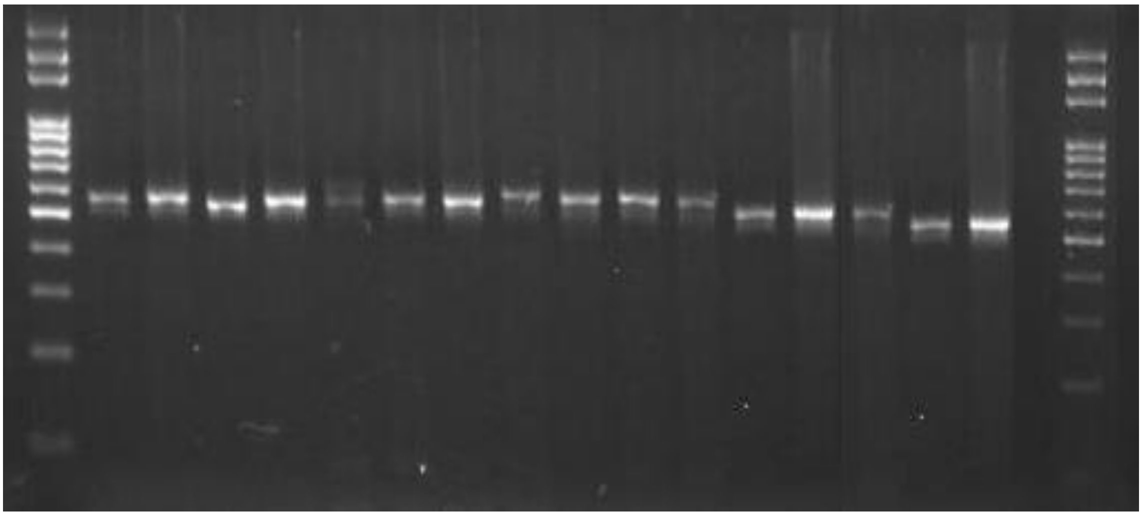

Figure 2. Electrophoresis in 2% agarose gel of ITS1/ITS4 amplified products for the fungal isolates
1 - *MR2*; **2 -** *Tr*; **3 -** *P3*; **4 -** *Pg*; **5 -** *D1*; **6 -** *Av*; **7 -** *Px*; **8 -** *P1*; **9 -** *P2*; **10 -** *Piz*; **11 -** *Rx*; **12 -** *SS*; **13** - *An*; **14 -** *F1*; **15 -** *F2*; **16** - *F3*; **M -** negative control; **L -** GeneRuler 100 bp Plus DNA Ladder.

Some authors claim that a single amplification by PCR-ITS only allows the identification/differentiation of fungal isolates to a small extent at the species level (Diguță et al., 2011). The obtained PCR products were subjected to separate digestion with the restriction enzymes *Sdu*I and *Hinf*I. A third *Mse*I or *Hae*III enzyme was used to complete species-level identification. The resulting fragments from the enzymatic cleavage were visualized in agarose gel electrophoresis and are shown in Figures 3 and 4.

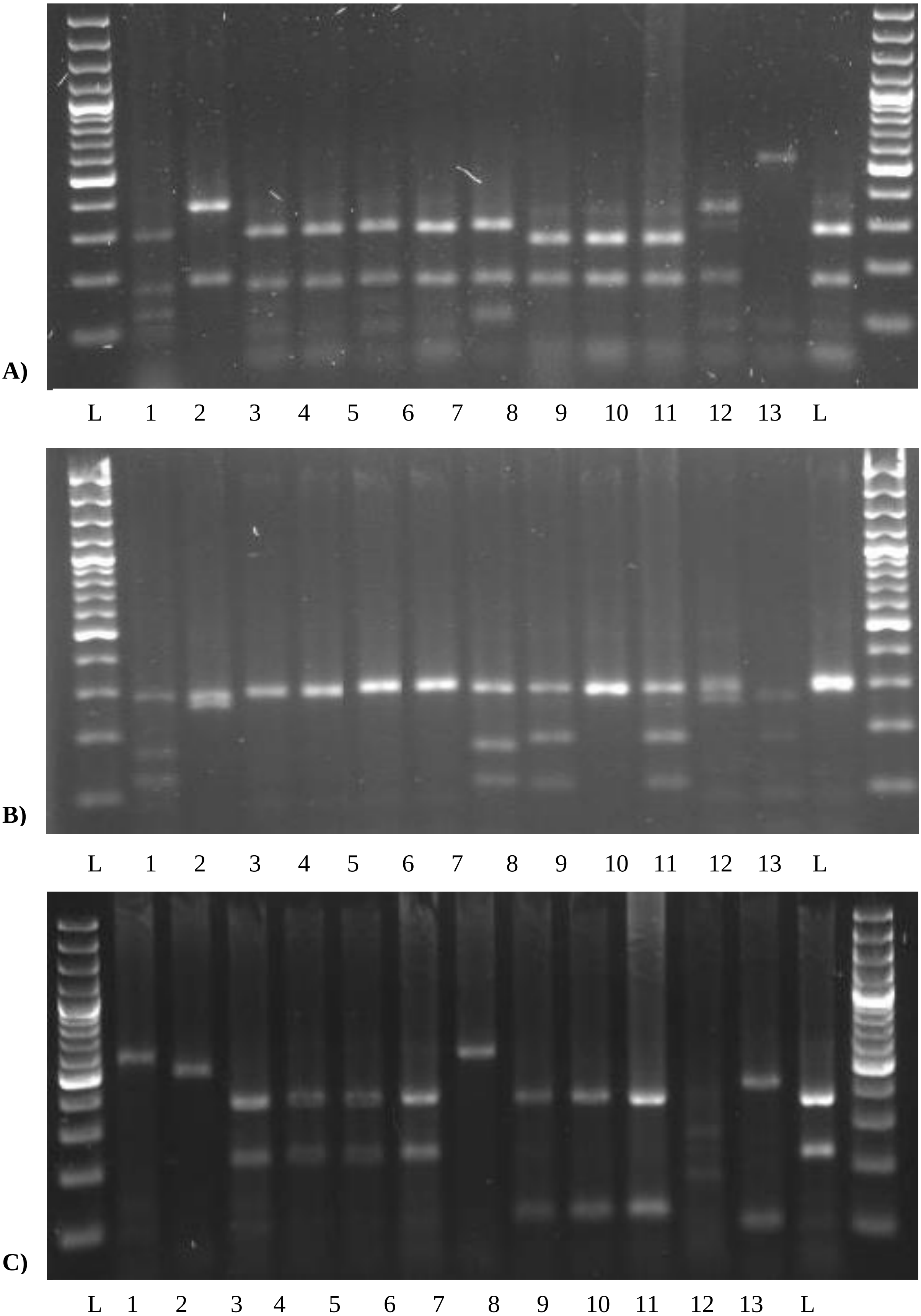

Figure 3. Restriction profile of the studied fungal isolates, after restriction endonucleases digestion**:**
A) *Sdu*I; B) *Hinf*I; C) *Mse*I
1 - *MR2*; **2 -** *Tr*; **3 -** *P3*; **4 -** *Pg*; **5 -** *D1*; **6 -** *Av*; **7 -** *Px*; **8 -** *P1*; **9 -** *P2*; **10 -** *Piz*; **11 -** *Rx*; **12 -** *SS*; **13** - *An;*
L - GeneRuler 100 bp Plus DNA Ladder

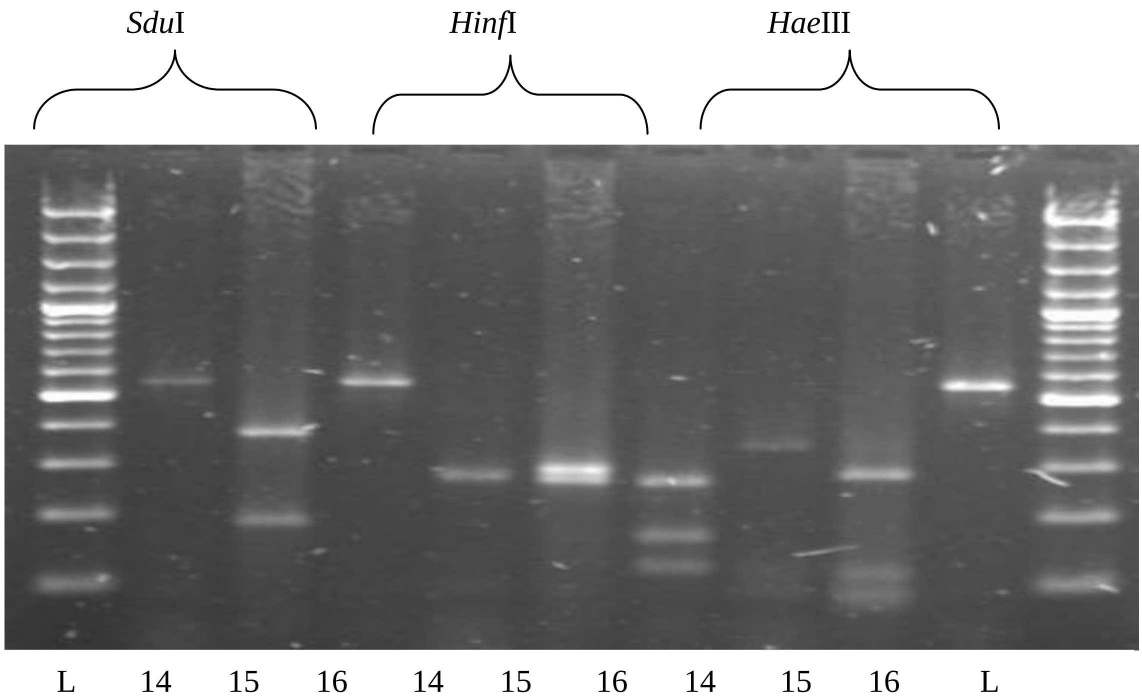

Figure 4. Fusarium spp. restriction profile after digestion with restriction endonucleases *Sdu*I; *Hinf*I; *Hae*III
14 - *F1*; **15 -** *F2*; **16 -** *F3*; **L -** GeneRuler 100 bp Plus DNA Ladder.

The restriction profiles obtained with each restriction enzyme are summarized in Table 1.

Table 1. Identification with PCR-ITS-RFLP of studied isolated fungi

Nr. crt.	Fungal isolate	Restriction fragments (bp)				Identification
		*Sdu*I	*Hinf*I	*Mse*I	*Hae*III	
1.	MR2	110 170 300	110 170 300	560		*Aspergillus versicolor*
2.	Tr	190 400	280 300	500		*Trichoderma viride*
3.	P3	170 300	300 300	210 370		*Aspergillus flavus*
4.	Pg	170 300	300 300	210 370		*Aspergillus flavus*
5.	D1	170 300	300 300	210 370		*Aspergillus flavus*
6.	Av	170 300	300 300	210 370		*Aspergillus flavus*
7.	Px	110 170 300	110 170 300	560		*Aspergillus versicolor*
8.	P1	170 270	110 190 300	110 370		*Penicillium expansum*
9.	P2	170 270	300 300	110 370		*Penicillium chrysogenum*
10.	Piz	170 270	110 190 300	110 370		*Penicillium expansum*
11.	Rx	170 340	280 280	180 280		*Aspergillus ruber*
12.	SS	540	180 280	110 430		*Botrytis cinerea*
13.	An	170 300	300 300	210 300		*Aspergillus niger*
14.	F1	560	280 280		100 120 360	*Fusarium tricinctum*
15.	F2	180 380	280 300		80 110 280	*Fusarium proliferatum*
16.	F3	560	120 170 280		560	*Fusarium* sp.

The resulting restriction profiles were compared with the restriction profiles of the sequences available in the theoretically obtained NCBI database.

Among *Aspergillus* sp. identified in this study, four isolates showed a specific profile of *A. flavus*, two isolates a specific profile of *A. versicolor*. One isolate was identified as *A. niger* and one isolate as *A. ruber*. Similar results regarding the dominance of the genus *Aspergillus* in the stored wheat samples were also presented in the studies of some Moroccan and Indian authors (Hajjaji et al., 2006; Kumari and Ghosh, 2016). Also the results of Jedidi et al. (2018) established that the wheat samples were contaminated especially with *Aspergillus* but also with *Alternaria* and *Eurotium*. However, Algerian researchers have also identified *A. orchraceus* (Riba et al., 2008).

Ahmad et al. (2014) differentiated *A. flavus*, known as a aflatoxin producer, from *A. parasiticus* based on the restriction profiles obtained with the *Ban*I and *Nla*IV enzymes of the amplified product from the *aflP* gene. Also, Somashekar et al. (2004) differentiated *A. flavus* from *A. parasiticus* based on restriction profiles of aflR amplicons with the enzyme PvuII. Specific detection of aflatoxin molds was performed on wheat samples using specific primers for the internally transcribed region (ITS1-5.8S-ITS2) (González-Salgado et al., 2009; Sardinãs et al., 2010).

The second major fungal genus was *Penicillium*. For the differentiation of *Penicillium* isolates, the restriction profiles obtained were compared with those obtained by Diguță et al. (2011). According to the results presented in Table 1, two isolates were identified as *P. expansum* and one isolate had a specific profile for *P. chrysogenum/P. crustosum/P. commune*. In this case, the *Cfr*9I endonuclease was used to complete the identification, and the resulting restriction profile corresponds to the *P. chrysogenum* species. Dupont et al. (2006) developed a PCR-RFLP method using a combination of 5 endonucleases that allowed the differentiation of 12 species of *Penicillium* from the *Biverticillium* subgenus. In another study, Diguță et al. (2011) optimized a PCR-ITS-RFLP method for the identification and differentiation of 22 different species out of a total of 24 Penicillium species detected on grape berries, with the exception of *P. thomii* and *P. glabrum* which remained non-differentiable. Also, Rousseaux and Guilloux-Bénatier (2016) using the PCR-RFLP technique were able to differentiate 22 species of the genus *Penicillium*. Previous studies (Kumari and Ghosh, 2016) also reported the existence of fungal species of the genera *Aspergillus* and *Penicillium* in stored wheat samples.

The restriction profiles analysis for the 3 isolates belonging to the genus *Fusarium*, obtained after enzymatic digestion with *Sdu*I, *Hinf*I and *Hae*III, allowed the identification of *F. tricinctum* and *F. proliferatum* species (based on the sequences already existing in the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/nuccore/). For the final identification of the F3 isolate, an analysis of the restriction profiles of other *Fusarium* species will be made, and the validation of the results will be done by sequencing. Llorens et al. (2006) amplified the IGS gene followed by digestion with 6 restriction enzymes (*Cfo*I, *Alu*I, *Hap*II, *Xho*I, *Eco*RI and *Pst*I) to differentiate 6 species of *Fusarium* (*F. culmorum, F. graminearum, F cerealis, F. poae, F. oxysporum,* and *Gibberella fujikuroi*). The results are consistent with those of the authors Sudharsan et al. (2017). Studies by Kachuei et al. (2015) showed that by using the restriction enzymes *Hha*I, *Msp*I, several species of *Fusarium,* such as *F. dimerum, F. sublunatum, F. beomiform, F. equiseti* and *F. compactum* were differentiated. They also used *Msp*I and *Taq*I enzymes to distinguish the following *Fusarium* species: *F. sacchari, F. brevicatenulatum, F. concolor* and *F. ambrosium*.

In this study, the most fungal strains identified by PCR-ITS-RFLP belong to the genus *Aspergillus* (50% of total fungal isolates), followed by *Fusarium* (19%) and *Penicillium* (19%). Also, were identified two isolates belonging to the species *Botrytis cinerea* and *Trichoderma* sp., respectively. The detection of these fungal species is of major importance, as most isolated species are potentially producing mycotoxins such as: aflatoxins, fumonisins, ochratoxin A, zearalenone and deoxynivalenol which represent a serious problem worldwide (FAO, 2004), causing many negative effects on human and animal health.

The detection of different species belonging to the genera *Aspergillus* and *Penicillium* can be explained by improper storage of cereals (high humidity, fluctuating temperatures, lack of aeration), which favors the growth of these molds known as storage molds. *Aspergillus* sp. has been shown to be the most common causative agent of grain rot, with *A. flavus* being the most predominant species detected. *Penicillium expansum* has been frequently detected on fruits (apples, grapes) (Diguţă et al., 2011) and only occasionally in cereals.

Fusarium is one of the major toxin-producing genera, such as fumonisins and trichothechechenes. *Fusarium* sp. are molds that frequently contaminate grains worldwide (Sampietro et al., 2011; Jedidi et al., 2018). *Fusarium* sp. are considered plant pathogens and are responsible for infections before and after harvest, however, some species of *Fusarium* are able to persist in harvested and stored grain and grow in warehouses when humidity conditions become favorable. *F. graminearum,* considered the main contaminant of wheat grains, was not detected in this study. At the same time, the studies of Sadhasivam et al. (2017) claim that the application of the multiplex PCR method on wheat samples stored in warehouses led to the detection of *Aspergillus* and *Fusarium* genera especially.

CONCLUSIONS

RFLP-PCR methods have been successfully applied for the isolation and identification of pathogenic fungi that contaminate stored wheat, having the advantage of being fast and specific**.**

The fungal strains identified by PCR-ITS-RFLP belong to the genus *Aspergillus* (50% of total fungal isolates), followed by *Fusarium* (19%) and *Penicillium* (19%).

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