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MOLECULAR DETECTION OF BLACK *ASPERGILLUS* AND *PENICILLIUM* SPECIES FROM DEALU MARE VINEYARD

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Abstract

The aim of this study was to determine the incidence and diversity of black Aspergillus and Penicillium species on white and red grape varieties in Dealu Mare vineyard (in two vilticultural centres - Valea Calugareasca and Pietroasa), during the harvest time of 2014 and 2015. A total of 61 fungal strains belonging to black Aspergillus (28 strains) and Penicillium (33 strains) were isolated. An RFLP analysis of the 5.8 S-ITS region was performed by using different combinations of endonucleases for molecular identification of fungal isolates at species level. A. niger (accounting 60.71% of all isolates) and P. expansum (66.66%) were the predominant species identified, followed by A. tubingensis (17.85%) and P. chrysogenum (21.21%). Higher biodiversity of fungal isolates has been found in Pietroasa Centre than from Valea Calugareasca.

Key words*: black Aspergillus, Penicillium sp., PCR-ITS RFLP, grapes, Dealu Mare vineyard.*

INTRODUCTION

Filamentous fungi (or molds) are communally contaminants of grapes. In recent years, particular attention has been drawn of black *Aspergillus* and *Penicillium* on grapes, being considered the main spoilage agents of grapes and grape-derived products and is responsible for significant economic losses. Rousseaux et al. (2014) reviewed thirty-six species of *Aspergillus* and fifty-nine different species of *Penicillium* identified on grapes in vineyards around the world.

The incidence of the species of black *Aspergillus* and *Penicillium* has been studied on grape varieties from vineyards worldwide: France (La Guerche et al., 2004; Garcia et al., 2006; Bejaoui et al., 2006; Diguță et al., 2011; Ahmed et al., 2015); Portugal (Serra et al., 2006); Spain (Martínez-Culebras and Ramón, 2007; Sardinas et al., 2011; Sempere et al., 2011; Garcia-Cela et al., 2014); Italy (Ayoub et al., 2010; De Rossi et al., 2011; Spadaro et al., 2012; Somma et al., 2012); Greece (Kizis et al., 2014; Kogkaki et al., 2015); Slovakia (Felsociova et al., 2013; Santini et al., 2014; Felsociova et al., 2015); Romania (Diguță et al., 2011; Diguță et al., 2015; Diguță et al., 2016).

Also, several species of *Aspergillus* section *Nigri* and *Penicillium* are recognized as potentially producers of mycotoxins (ochratoxin A, patulin etc.) and volatile compounds (geosmin, 2-methyl-isoborneol etc.) (La Guerche et al., 2004; Garcia et al., 2006; Martinez-Culebras and Ramon, 2007; Somma et al., 2012; Rousseaux et al., 2014). Among the black aspergilli, *A. carbonarius* has been considered as the main OTA producer (Belli et al., 2006), followed by species belonging *A. niger* aggregate (*A. niger* and *A. tubingensis*) (Bejaoui et al., 2006; Perrone et al., 2006). Among *Penicillium* species, *P. expansum* is the major causal agents of the blue mold rot of grapes and the main source of volatile compounds production which determinate off-flavours (La Guerche et al., 2004, Garcia et al., 2006).

The detection of mycotoxins and volatile compounds in grape wines can influence not only detrimental to the sanitary quality, but also to the organoleptic quality (La Guerche et al., 2004; Garcia et al., 2006; Martinez-Culebras and Ramon, 2007; Somma et al., 2012; Rousseaux et al., 2014). In Romania, Geana et al. (2012) reported the detection to traceable OTA levels (ranging from 0.06 ng/mL to 0.45 ng/mL) in 7 wine commercialized on the

Romanian market, however, the concentrations found being far below the proposed European limit (2 ng/mL). Black aspergilli and *Pencillium* species are difficult to taxonomical identification by classical methods. Among molecular approaches, restriction fragment length polymorphism (RFLP) based on ITS-5.8S region have been proved useful tool to the identification and classification the species belonging to *Aspergillus* section *Nigri* (Accensi et al., 1999; Bau et al., 2006; Martínez-Culebras and Ramón, 2007; Kizis et al., 2014); to differentiate *P. expansum* among fungal grape species (Garcia et al., 2006); to discriminate 22 species belonging to *Penicillium* genus and 7 species belonging to *Aspergillus* (Diguţă et al., 2011).

The latest available data of The International Organisation of Vine and Wine (OIV) shows that Romania is considered in top viticultural production potential with an area under vines the 191 thousands of hectares of overall in European vineyards (OIV, 2017a). After two poor harvests, Romania returned to a high level of production (5.3 millions of hectoliters) (OIV, 2017b).

Consequently, there is a special need to manage and combat the spoilage molds directly in the vineyard in order to obtain high quality of the Romanian wines which can compete with high quoted wines coming from other traditional wine-making countries (France, Italy, Spain etc.) or more recent producers as USA, Argentina or South Africa.

In this context, this study was focused to determinate the incidence and diversity of black *Aspergillus* and *Penicillium* species on white and red grape varieties in Dealu Mare vineyard (in two vilticultural centres - Valea Calugareasca and Pietroasa), during the harvest time of 2014 and 2015.

MATERIALS AND METHODS

Fungal isolation

The grape samples (white and red grape varieties) were randomly and aseptically collected from Dealu Mare vineyard (in two vilticultural centres - Valea Calugareasca and Pietroasa) at harvest time in 2014 and 2015 season (Table 1). Fungal isolation was performed according to the method described by Diguţă et al. (2011). The suspension was serially diluted and dilutions were plated on Dichloran Rose-Bengale Agar (Mecconti, Poland) supplemented with Chloramphenicol. Plates were incubated at 25°C in the dark for 5-7 days. Only fungal strains considered to represent black *Aspergillus* and *Penicillium* species were isolated and maintained to Potato dextrose agar (PDA, Mecconti, Poland). The pure cultures were preliminary screened by colony characteristics (mycelium type, color and growth type) as well as microscopic characteristics (conidiophores and spore) (Pitt and Hocking, 2009; Varga et al., 2011).

Table 1. Grape samples used in this study

Vilticultural centres	Grape variety	Acronym
Valea Călugărească	Fetească Regală (white grape)	FRV
	Cabernet Sauvignon (red grape)	CSV
Pietroasa	Fetească Regală Conventional (white grape)	FRCP
	Cabernet Sauvignon Conventional (red grape)	CSCONP
	Cabernet Sauvignon Ecologic* (red grape)	CEP
	Tămâioasă Românească Conventional (white grape)	TăRCP

*The vineyard is under certified ecological conditions.

DNA extraction

Fungal isolates were grown in 10 ml of PDB Broth (Mecconti, Poland) at 25ºC for 72 h. Mycelia were collected after centrifugation (10000 rpm for 5 minutes) and washed with sterile saline water then frozen at -25°C until use. Approximately 200mg of fungal biomass was used for DNA extraction by the use of ZR Fungal/Bacterial MiniPrep™ Kit (Zymo Research, USA), according to the manufacturer's instructions.

PCR-RFLP conditions

- Amplification of the 5.8S-ITS region was performed with universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3', forward) or ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3', forward) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3', reverse) in order to identify fungal strains (White et al., 1990). The PCR program for the amplification with ITS1/ITS4 was as follows: 2 min at 94°C, 34 cycles with 1 min at 94°C, 1 min at 55°C, 2

min at 72°C, with a final extension for 7 min at 72°C. The PCR program for the amplification with ITS5/ITS4 was as follows: 2 min at 95°C, 35 cycles consisting of 1 min at 95°C, 1 min at 52°C and 1 min at 72°C, with a final extension for 10 min at 72°C.

PCR products were digested with different combination of restriction enzymes *Cfr9*I, *Hpy*188I, *Hinf*I, *Hha*I, *Mse*I, *Nla*III, *Rsa*I and *Sdu*I (Thermo Scientific, USA), according to PCR-ITS-RFLP methods developed by Martínez-Culebras and Ramón (2007) and Diguţă et al. (2011) (Figure 1).

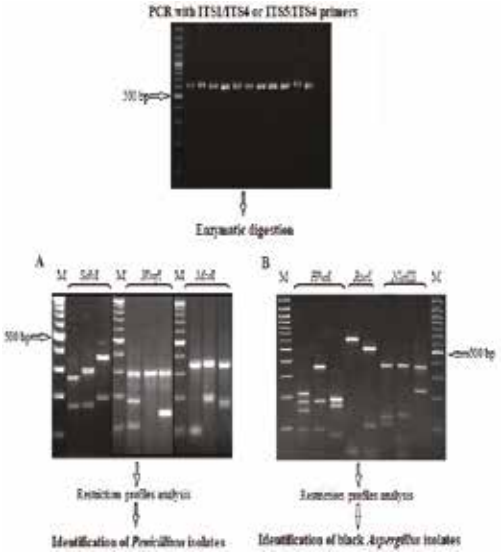

Figure 1. Identification of black *Aspergillus* and *Penicillium* isolates by PCR-RFLP based 5.8S-ITS region, adapted after Diguţă et al. (2011) (**A**) and Martinez-Culebras and Ramón (2007) (B); M - GeneRuler 100bp Plus DNA Ladder

PCR- RFLP analysis

The amplification and digestion results have been visualized under UV (254 nm) after electrophoresis in agarose gel 2% (90V/60 minutes). All fragment sizes were evaluated by the fluorescence intensity and were approximated using the DNA ladder (GeneRuler 100bp Plus DNA Ladder, Thermo Scientific, USA). Restriction fragments are considered to be distinguished when the difference between them is more than 20bp and considered to be fragments in common if migrated the same distance during agarose gel electrophoresis, respectively. Restriction fragments smaller than 70bp could not be clearly visualized and were not included in our results. Restriction patterns were compared with RFLP patterns obtained by Martínez-Culebras and Ramón (2007) and Diguţă et al. (2011).

RESULTS AND DISCUSSIONS

A total of 61 fungal strains including 28 strains black *Aspergillus* and 33 strains *Penicillium* were isolated in this study. All the isolates have been taxonomically identified by molecular methods using PCR-RFLP. Twenty eight fungal strains belonging to *Aspergillus* section *Nigri* have been isolated. The primer pairs ITS5/ITS4 were used to amplify the 5.8S-ITS region, followed by restriction enzyme digestion with three endonucleases *Hha*I, *Nla*III and *Rsa*I, according to ITS-RFLP method developed by Martínez-Culebras and Ramón (2007) (Table 2). Based on RFLP types, four species belonging to black *Aspergillus* have been identified: *A. niger aggregate (A. niger* and *A. tubingensis), A. aculeatus, A. japonicus* (Table 2).

A. niger (17 of all the isolates) was dominated among isolated fungus from black aspergilli, followed by *A. tubingensis* (5 isolates). Moreover, two strains belonging to the uniseriate species *Aspergillus aculeatus* and *A. japonicus* showed the same PCR-RFLP. Digestion with *Hinf*I showed two distinct restriction patterns: 110+180+270 (for *A. aculeatus*) and 270+290 (for *A. japonicus*) (personal data). Based on RFLP profile, 4 isolates have been identified as *A. japonicus.*

Thirty-three fungal strains belonging to *Penicillium* genus have been isolated. Amplification of the 5.8S-ITS region was performed with the universal primers ITS1/ITS4 followed by digestion with the combination of three endonucleases *Sdu*I, *Hinf*I and *Mse*I to discriminate *Penicillium* isolates at species level, according to PCR-ITS-RFLP method developed by Diguta et al. (2011) (Table 3).

According to Table 3, of the *Penicillium* isolates, 22 isolates showed a RFLP profile corresponding to *P. expansum*. Seven isolates have been identified as *P. chrysogenum*. This

result has been confirmed with other endonucleases *Cfr9*I or *Hpy*188I to differentiate *P. chrysogenum* from *P. crustosum* and *P. commune* (personal data). In addition, other 4 isolates of *Penicillium* sp. showed new RFLP profiles. To validate our results, sequencing is required.

The distribution of identified species on grapes varieties in Dealu Mare vineyard is given in Figure 2. Higher biodiversity of fungal isolates has been found in Pietroasa Centre than from Valea Calugareasca (Figure 2 A and B).

The distribution of *Aspergillus* sp. and *Penicillium* sp. on the grape varieties can vary from one vineyard to another, from one year to another, grape maturity, climatic conditions and viticultural practices being key factors, which leads to difficulty in generalizing the management and combating of these molds directly from the vineyard. However, the impacts of climate changes and viticultural practices on the occurrence of black *Aspergillus* and *Penicillium* on grapes have been not taken into account, when have been analysed the results obtained in this study.

According to figure 2 we found that *A. niger* was the predominant species isolated from grapes. *A. carbonarius*, the main species of ochratoxigenic species, have not been isolated. However, in another study, *A. carbonarius* has been detected and quantified by qPCR in all naturally grape samples tacking in account in this study (Diguță et al. 2016).

Table 2. RFLP analysis of 5.8S-ITS region exhibited by the *Aspergillus* isolates

No.	*Aspergillus* sp.	Number isolates	Restriction fragments		
			*Hha*I (pb)	*Nla*III (pb)	*Rsa*I (pb)
1.	*A. niger*	17	90 120 180 210	110+130+360	520
2.	*A. tubingensis*	5	90 120 180 210	110 130 360	80+480
3.	*A. aculeatus*	1	70 140 180 180	220+350	80+480
4.	*A. japonicus*	4	70 140 180 180	220 +350	80 +480

Table 3. RFLP analysis of 5.8S-ITS region exhibited by the *Penicillium* isolates

No.	*Penicillium* sp.	Number isolates	Restriction fragments		
			*Sdu*I (pb)	*Hinf*I (pb)	*Mse*I(pb)
1.	*P. expansum*	22	170 270	110 180 290	110 110 360
2.	*P. chrysogenum*	7	170 270	290 290	110 110 360
3.	*Penicillium* 1	1	170 270	110 180 290	560
4.	*Penicillium* 2	1	170 270	290 290	560
5.	*Penicillium* 3	1	190 320	290 290	110 110 360
6.	*Penicillium* 4	1	560	130 160 290	190 240

On the Fetească Regală grape variety (in 2014) and Cabernet Sauvignon (in 2015), no strain of *Aspergillus* sp. or *Penicillium* sp. have been isolated (Figure 2A). Other species commonly found on grapes, namely *Alternaria alternata, Cladoporium cladosporioides, Epicoccum nigrum* on both varieties were identified (personal data). A certain fungal diversity was observed on Fetească Regală grape variety (in 2015), predominantly *P. expansum*, followed by other new *Penicillium* species, A. *tubingensis* (Figure 2A).

A special attention has been given to fungal mycobiota of two autochthonous grape varieties (Fetească Regală and Tămâioasă Românească) and international grape variety Cabernet Sauvignon from Pietroasa Centre (Figure 2B). Diversity of *Aspergillus* sp. and *Penicillium* sp. is higher from Tămâioasă Românească variety (TăRCP - for 2014) and Fetească Regală variety (FRCP - for 2014 and 2015) from Pietroasa Centre (Figure 2 B). *P. expansum* was detected on FRCP (both years season), TăRCP (2014) and CSCONP (2015). Also, *P. chrysogenum* was another highly isolated species on the two grape varieties analyzed (TăRCP, for 2014 and FRCP, for 2015). *A. niger* was isolated predominantly on

the Cabernet Sauvignon Conventional Variety (CSCONP) in 2014 season. Also, *A. niger* was identified on the Cabernet Sauvignon Ecological (CSEP) variety in 2015. *A. tubingensis* was predominated CSEP (2014) and TăRCP (2015) (Figure 2B).

Two other uniseriate *Aspergillus* species, *A. japonicus* and *A. aculeatus* were punctually isolated on CSCONP (both seasons), TăRCP (2014) and FRCP (2015) (Figure 2B).

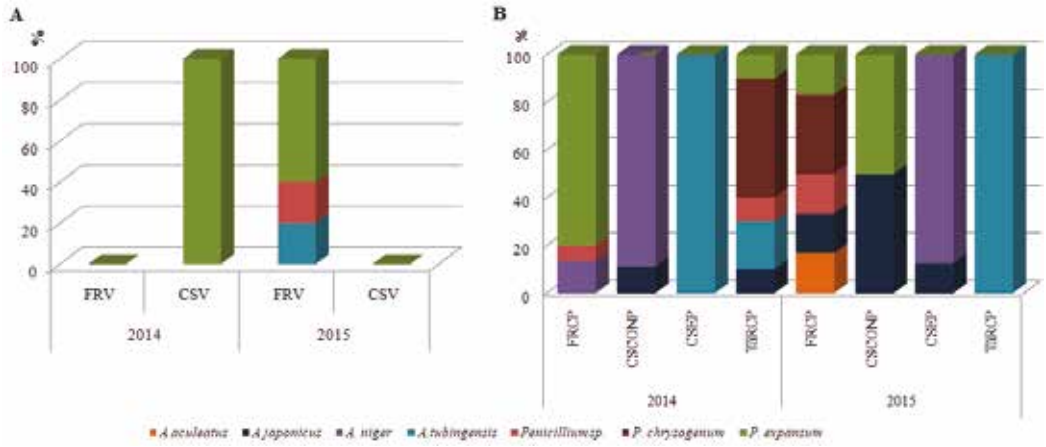

Figure 2. Distribution of black *Aspergillus* and *Penicillium* isolates in two vilticultural centres - Valea Călugărească (**A**) and Pietroasa Centre (**B**) on different grape varieties

CONCLUSIONS

A total of 61 fungal isolates have been isolated from red and white wines cultivated in Dealu Mare vineyard under conventional and ecological conditions.

A. niger and *P. expansum* were the predominant species identified among the isolates. No significant difference in fungal biodiversity of the grapes has been detected between conventional and ecological cultures.

In our work four fungal isolates couldn't be clearly identified because of the lack of information in the enzymatic profiles in databases. However, PCR-RFLP can be a useful tool for the identification of different species belonging to black *Aspergillus* and *Penicillium*.

When this identification is performed in early stage, better prevention measures and controls can be performed for a good quality management of the final product, the wine.

Future work will be focused on mycotoxin and volatile compounds production of isolated strains.

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