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Rapid detection of *Aspergillus carbonarius* in Romanian vineyards

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Abstract

Grape spoilage and the biosynthesis of ochratoxin A (OTA) by Aspergillus carbonarius are the main problems which confronting the global wine industry. The aim of this study was to use qPCR tool to rapid detection and quantification of A. carbonarius in six native grape samples from two Romanian vineyards, during 2014 and 2015. A standard curve was developed by plotting the logarithm of known concentrations of A. carbonarius DNA against the cycle threshold (Ct) value. Efficiency value was 97.37% and the limit of detection was estimated to be 1.7 pg DNA. A. carbonarius was detected in all grape samples. qPCR assay proved to be selective and sensitive for rapid assessment of the potential ochratoxigenic infection in Romanian vineyards.

Keywords: *Aspergillus carbonarius, qPCR, Romanian vineyards*

1. Introduction

Aspergillus section *Nigri* (known black aspergilli) have been frequently isolated on grapes in vineyard around the world (reviewed by ROUSSEAUX & al. [1]). Only some species belonging to the black aspergilli produce mycotoxins. Ochratoxin A (OTA) is one the most significant mycotoxin detected on grapes and in wine (ZIMMERLI and DICK, [2]; CABANES & al., [3]; BAU & al., [4], BEJAOUI & al., [5]; EL KHOURY & al, [6]). OTA has been shown to have nephrotoxic, hepatotoxic, teratogenic and carcinogenic effects. The European Commission by the Regulation (EC) N°123/2005 (EC, [7]) has established 2 µg/L as the maximum admissible limit of OTA in wine in order to protect consumers. Among section *Nigri*, *A. carbonarius* has been reported to display the highest ochratoxigenic potential (SAGE & al., [8]; ABARCA & al., [9]; BEJAOUI & al., [5]; PERRONE & al., [10]). Thus, the monitoring of *A. carbonarius* is essential in the vineyard, in order to prevent/combat the contaminated grapes. As an alternative to traditional identification techniques, PCR-based methods are rapid, sensitive, specific and allow an accurate identification and discrimination of fungal species. Different molecular techniques such as polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) (ACCENSI & al., [11]; MEDINA & al., [12]; BAU & al., [13], MARTÍNEZ-CULEBRAS and RAMÓN, [14]; DIGUȚĂ & al., [15]), randomly amplified polymorphic DNA (RAPD) (BAU & al., [4]; DACHOUPAKAN & al., [30]), amplified fragment length polymorphisms (AFLP) and fluorescent AFLP (fAFLP) (PERRONE & al., [16]; OLIVERI & al., [17]) were used to identify and distinguish among the main species of black aspergilli or to identify ochratoxin A-producing black *Aspergillus* isolates. However, the main disadvantage is that these techniques can be applied to fungal identification after culture and isolation on culture media. In the last years, culture-independent methods

have been developed. Among these methods, real-time or quantitative PCR (qPCR) using different chemistries, such as SYBR® Green I dye and TaqMan® have been developed and shown to be useful for the detection and quantification of *Aspergillus carbonarius* on grapes (MULÈ & al., [18]; ATOUI & al., [19]; SELMA & al., [20]; GONZÁLEZ-SALGADO & al. [21]; SPADARO & al., [22]; CASTELLA and CABAÑES, [23]) or for the simultaneous detection of *A. niger* aggregate and *A. carbonarius* (SELMA & al., [24]) and the specific detection of *A. carbonarius* and *A. niger*, even in symptomless grape samples (AYOUB & al., [25]). The aim of this study was to apply a qPCR technique for the rapid and specific detection *A. carbonarius* in Romanian vineyards.

2. Materials and methods

Grapes sampling procedure

Six native grape samples (white and red grape varieties) were taken during full maturity stage from two consecrated Romanian vineyards, in two consecutive harvests, 2014 and 2015 respectively. For each plot, dozen bunches of grapes were cut at random using shears sterilized with ethanol. The bunches were collected in sterilized plastic bags without any hand contact and placed in a cooler at 4°C until laboratory analysis. Two hundred of berries were harvested in sterilized flasks in aseptic conditions. Two hundred milliliters of 0.9% sterile NaCl solution containing 0.2% Tween 80 were added to the berries. This mix was shaken for 1 hour to pull of the micro-organisms in suspension.

Table 1. Grape sample used in the study

Romanian Vineyards	Grape variety	Acronym
Pietroasa	Fetească Regală (white grape)	FRC
	Cabernet Sauvignon (red grape)	CCON
	Ecologic Cabernet Sauvignon* (red grape)	CE
	Tămâioasă Românească (white grape)	TăRC
Valea Călugărească (Dealu Mare)	Fetească Regală (white grape)	FRV
	Cabernet Sauvignon (red grape)	CSV

* The vineyard is under certified ecological conditions

Reference strain and growth conditions

A. carbonarius MUCL44624 (Mycothéque de l'Université Catholique de Louvain, Belgium) was used in this study as reference strain. The strain was grown on PDA at 25°C for 2 weeks and collected from the agar plate using sterile distilled water containing 0.05% (w/v) Tween 80. For DNA extraction, the number of spores was counted with the aid of a haemacytometer and adjusted to 10^9 spores.mL^{-1}.

DNA extraction

DNA extraction from *A. carbonarius* spores and washing grape suspension was performed using a fungal DNA kit (ZR Fungal/Bacterial DNA MiniPrep, Zymo Research). 2mL of spores solutions or 2mL of the washing solution were centrifuged at 10 000 g for 20 min. The pellet was used for DNA extraction, according to the manufacturer's instructions. Finally, DNA was resuspended in 50 µL free-nuclease-water.

Real-time PCR amplification

Specific *A. carbonarius* primers targeting from two non-conserved sequences in the AT domain of *Ac12RL3* (polyketide synthase gene) reported by ATOUI & al. [19] were used:

Ac12RL_OTAF (5'-AATATATCGACTATCTGGACGAGCG-3') and Ac12RL_OTAR (5'-CCCTCTAGCGTCTCCCGAAG-3'). All primers were purchased from Invitrogen (Cergy, France). The DNA sample (5 µL) was mixed in a final volume of 25 µL with *A. carbonarius* primer mixture containing 0.56 µM of either, with separate 0.05 µL ROX vial, 2x I QTMSYBR Green supermix (Maxima SYBR Green qPCR Master Mix (Thermo Scientific) or water. Reactions were performed in ABI PRISMR 7900 HT apparatus (Applied Biosystems, Foster City, CA, USA). We used a program of: 15 min at 95°C, followed by 40 cycles of 15 s at 95°C and 30 s at 58°C and 72 °C for 30 s. To generate the standard curve, a 10-fold dilutions (1.7 ng to 1.7 pg) of total *A. carbonarius* DNA (MUCL 44624) whose concentration was previously determined spectrophotometically, were subjected to qPCR under the same conditions described above. The results obtained are the mean of three independent experiments. The cycle threshold (Ct), or the PCR cycle where fluorescence first occurred, was determined automatically using Applied software after setting the baseline to 100. The efficiency (*E*) of the PCR assay was calculated using the formula, $E = [10^{-1/slope}-1] \times 100$, where the slope was extracted from the curve Ct = f(log Q0) and Q0 is the initial DNA in the assay. *E* was expressed as percentage.

3. Results and discussions

During the last years, real-time or quantitative PCR (qPCR) has been developed and shown to be useful in monitoring and quantification of ochratoxigenic fungi on artificially contaminated grapes (SELMA & al., [20], [24]; GONZÁLEZ-SALGADO & al. [21]; SPADARO & al., [22]; CASTELLA and CABAÑES, [23]) or in native grapes samples (ATOUI & al., [19]). The pair of primers (Ac12RL_OTAF and Ac12RL_OTAR) previously reported by ATOUI & al., [19] has been taking into account for this study.

To determine the sensitivity and detection limit of the qPCR, DNA obtained from *A.carbonarius* at a concentration of 10^9 spores.ml^{-1} was serially diluted 10-fold and amplified in the conditions previously described.

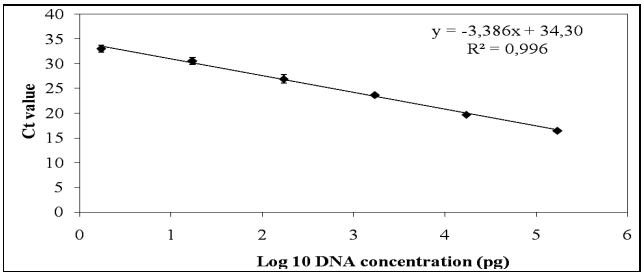

Figure 1. Standard curve obtained from the amplification of 10-fold dilutions of target genomic *Aspergillus carbonarius* DNA *vs*. threshold cycle (Ct)

A standard curve was generated by plotting Ct value *vs*. the $\log_{10}$ of DNA (pg) (Figure 1). The lower detection limit is the minimal of genomic *A. carbonarius* DNA that can be detected by this method. The high linear correlation coefficient ($R^2 = 0.996$) was observed across the whole range used. The slope of the standard curve was -3.386. The efficiency value was 97.37%. Under conditions previously described, the maximum Ct value that could be used was 34, which corresponds to a DNA concentration of 1.7 pg (Figure 1). The accuracy obtained was in the same range as the qPCR assay described by ATOUI & al. [19].

Application of the qPCR technique to quantify A. carbonarius in native grape samples

In this study, we used the qPCR method to assess the level of *A. carbonarius* in six native grape samples from two Romanian vineyards. The amount of *A. carbonarius* DNA detected in an unknown sample was obtained by interpolating its treshold cycle values against the standard curve. *A. carbonarius* was detected in all naturally grape samples and was higher (log 4.6-5.0 pg) than established detection limit (Figure 2). Previous studies have shown that geographic area, grape variety, meteorological conditions, fungicides treatments and prophylactic methods applied at different growing stages can influence the variation of *Aspergillus* section *Nigri* incidence on grapes (ROUSSEAUX & al. [1]; BAU & al. [4]; BEJAOUI & al. [5]; SAGE & al. [8]; MEDINA & al. [12]; BAU & al. [13]; BELLÍ & al. [31]). In our study, the *A. carbonarius* DNA amounts for different harvest years were slightly different, with higher values for the harvest 2014 against 2015.

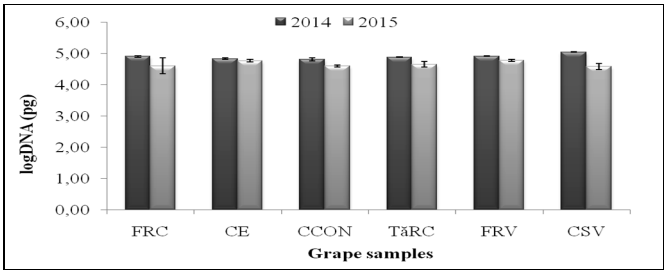

Figure 2. DNA concentration of *Aspergillus carbonarius* on each trial grape berries

Related to the grape color, it has not been noticed a significant difference between the detection of *A. carbonarius* on white grape varieties and red grape varieties (Figure 2). *Aspergillus* isolates belonging of *Aspergillus* section *Nigri* has been detected and identified by PCR-ITS RFLP method (unpublished data). However, no strain of *Aspergillus* isolated was identified as *Aspergillus carbonarius*. The applicability of this assay for the wine industry provides a good tool for early detection and quantification of others two spoilage molds (*Botrytis cinerea* and *Penicillium expasnum*) on grape berries. qPCR assay has been developed for monitoring *Botrytis cinerea* infection (CADLE-DAVIDSON, [26]) or quantitative analysis of *B. cinerea* on artificially contaminated table grapes (CELIK & al. [27]). DIGUTA & al. [28] has been developed a specific qPCR method to detect *B. cinerea* in vineyards and utilized to compare the effects of various treatment strategies including various fungicide treatments. More recently, BACHA & al., [29] has been developed a qPCR assay to monitoring of *P. expansum* and *P. restrictum* (geosmin-producing fungi) in grapes.

4. Conclusions

In this work we used qPCR assay to the detection and quantification of *Aspergillus carbonarius* in six native grape samples without visible symptoms of rotting from two Romanian vineyards. Results obtained have been shown a good relation between logarithm of *A. carbonarius* genomic DNA and threshold cycles over the range of DNA concentrations used. The presented method can confirm in a short time the detection and quantification of *A. carbonarius* in grapes in about 8 h (including grapes sampling procedure, DNA extraction and qPCR assay). qPCR assay could help the early and rapid detection and quantification of

A. carbonarius and could help winemakers to take the good and rapid decisions in order to prevent ochratoxin A contamination in wine and grapes.

5. Acknowledgements

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References

1. ROUSSEAUX S, DIGUTA CF, RADOÏ-MATEI F, ALEXANDRE H, GUILLOUX-BÉNATIER M, Non-*Botrytis* grape-rotting fungi responsible for earthy and moldy off-flavors and mycotoxins (REVIEW), *Food Microbiology*, 38, 104-121, (2014).
2. ZIMMERLI B and DICK R, Ochratoxin A in table wine and grape-juice: occurrence and risk assessment, *Food Additives and Contaminants,* 13(6), 655-668, (1996).
3. CABANES FJ, ACCENSI F, BRAGULAT MR, ABARCA ML, CASTELLA G, MINGUEZ S, PONS A, What is the source of ochratoxin A in wine? *International Journal of Food Microbiology,* 79(3), 213-215, (2002).
4. BAU M, BRAGULAT MR, ABRACA ML, MINGUEZ S, CABAÑES FJ, Ochratoxigenic species from Spanish wine grapes. *International Journal of Food Microbiology,* 98, 125-130, (2005).
5. BEJAOUI H, MATHIEU F, TAILLANDIER P, LEBRIHI A, Black aspergilli and ochratoxin A production in French vineyards. *International Journal of Food Microbiology,* 111, S46-S52, (2006a).
6. EL KHOURY A, RIZK T, LTEIF R, AZOURI H, DELIA ML, LEBRIHI A, Fungal contamination and aflatoxin B1 and ochratoxin A in Lebanese wine–grapes and musts. *Food and Chemical Toxicology* 46: 2244-2250, (2008).
7. EUROPEAN COMMISSION, Commission regulation (EC) N_ 123/2005 of 26 January 2005 amending regulation (EC) N_ 466/2001 as regards ochratoxin A. *Official Journal of the European Union*, L25, 3-5, (2005).
8. SAGE L, GARON D, SEIGLE-MURANDI F, Fungal microflora and ochratoxin a risk in French vineyards. *Journal of Agricultural and Food Chemistry*, 52(18), 5764-5768, (2004).
9. ABARCA ML, ACCENSI F, BRAGULAT MR, CASTELLA G, CABANES FJ, *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried wine fruits from the Spanish market. *Journal of Food Protection*, 66, 504-506, (2003).
10. PERRONE G, MULÈ G, SUSCA A, BATTILANI P, PIETRI A, LOGRIECO A, Ochratoxin A production and AFLP analysis of *Aspergillus carbonarius, Aspergillus tubingensis,* and *Aspergillus niger* strains isolated from grapes in Italy. *Applied and Environmental Microbiology*, 72, 680-685, (2006a).
11. ACCENSI F, CANO J, FIGUERA L, ABARCA ML, CABANES FJ, New PCR method to differentiate species in the *Aspergillus niger* aggregate. *FEMS Microbiology Letters*, 180, 191-196, (1999).
12. MEDINA A, MATEO R, LÓPEZ-OCAÑA L, VALLE ALGARRA FM, JIMÉNEZ, M, Study of Spanish grape mycobiota and ocratoxina A production by isolates of *Aspergillus tubingensis* and other members of *Aspergillus* section *Nigri*, *Applied and Environmental Microbiology*, 71, 4696-4702, (2005).
13. BAU M, CASTELLÁ G, BRAGULAT MR, CABAÑES FJ, RFLP characterization of *Aspergillus niger* aggregate species from grapes from Europe and Israel. *International Journal of Food Microbiology*, 111, S18-S21, (2006).
14. MARTINEZ-CULEBRAS PV, RAMON D, An ITS-RFLP method to identify black *Aspergillus* isolates responsible for OTA contamination in grapes and wine. *International Journal of Food Microbiology*, 113, 147-153, (2007).

15. DIGUTA CF, VINCENT B, GUILLOUX-BENATIER M, ALEXANDRE H, ROUSSEAUX S, PCR ITS-RFLP: a useful method for identifying fungal isolates on grapes, *Food Microbiology,* 28(6), 1145-1154, (2011).
16. PERRONE G, SUSCA A, EPIFANI F, MULÈ G, AFLP characterization of Southern Europe population of *Aspergillus* sect. *Nigri* from grapes. *International Journal of Food Microbiology*, 111, S22–S27, (2006b).
17. OLIVERI C, TORTA L, CATARA V, A polyphasic approach to the identification of ochratoxin A-producing black *Aspergillus* isolates from vineyards in Sicily. *International Journal of Food Microbiology*, 127, 147-154, (2008).
18. MULÈ G, SUSCA A, LOGRIECO A, STEA G, VISCONTI A, Development of a quantitative real-time PCR assay for the detection of *Aspergillus carbonarius* in grapes. *International Journal of Food Microbiology*, 111, S28-S34, (2006).
19. ATOUI A, MATHIEU F, LEBRIHI A, Targeting a polyketide synthase gene for *Aspergillus carbonarius* quantification and ochratoxin A assessment in grapes using real-time PCR. *International Journal of Food Microbiology,* 115(3), 313-318, (2007).
20. SELMA VM, MARTINEZ-CULEBRAS PV, AZNAR R, Real-time based procedures for detection and quantification of *Aspergillus carbonarius* in wine grapes, *International Journal of Food Microbiolgy*, 122, 126-134 (2008).
21. GONZALEZ-SALGADO A, PATIÑO B, GIL-SERNA J, VAZQUEZ C, GONZALEZ-JAEN MT, Specific detection of *Aspergillus carbonarius* by SYBRs Green and TaqMans quantitative PCR assays based on the multicopy ITS2 region of the rRNA gene. *FEMS Microbiological Letters*, 295, 57-66, (2009).
22. SPADARO D, PATHARAJAN S, KARTIKEYAN M, LORÈ A, GARIBALDI A, GULLINO ML, Specific PCR primers for the detection of isolates of *Aspergillus carbonarius* producing ochratoxin A on grapevine, *Ann. Microbiol.,* 61, 267-272, 2010.
23. CASTELLA G, CABAÑES FJ, Development of a Real Time PCR system for detection of ochratoxin A-producing strains of the *Aspergillus niger* aggregate. *Food Control,* 22, 1367-1372, (2011).
24. SELMA MV, MARTÍNEZ-CULEBRAS PV, ELIZAQUIVEL P, AZNAR R, Simultaneous detection of the main black aspergilli responsible for ochratoxin A (OTA) contamination in grapes by multiplex real-time polymerase chain reaction. *Food Additives and Contaminants*, 26, 180-188, (2009).
25. AYOUB F., REVERBERI M, RICELLI A, D'ONGHIA, AM, YASEEN T, Early detection of *Aspergillus carbonarius* and *A. niger* on table grapes: a tool for quality improvement. *Food Additive Contamination*, 27, 1285-1293, (2010).
26. CADLE-DAVIDSON L, Monitoring pathogenesis of natural *Botrytis cinerea* infections in developing grape berries. *Am J Enol Viticult*, 59, 387–395, (2008).
27. CELIK M, KALPULOV T, ZUTAHY Y, ISH-SHALOM S, LURIE S, LICHTER A Quantitative and qualitative analysis of *Botrytis* inoculated on table grapes by qPCR and antibodies. *Postharvest Biology and Technology,* 52, 235-239, (2009).
28. DIGUTA CF, ROUSSEAUX S, WEIDMANN S, BRETIN N, VINCENT B, GUILLOUX-BENATIER M, ALEXANDRE H, Development of a qPCR assay for specific quantification of *Botrytis cinerea* on grapes, *FEMS Microbiology Letters,* 313(1), 81-87, (2010).
29. BACHA N, ECHARKI Z, MATHIEU F, LEBRIHI A, Development of a novel quantitative PCR assay as a measurement for the presence of geosmin producing fungi, *Journal of Applied Microbiology,* 118(5), 1144-115,1 (2015).
30. DACHOUPAKAN C, RATOMAHENINA R, MARTINEZ V, GUIRAUD JP, BACCOU JC, SCHORR-GALINDO S, Study of the phenotypic and genotypic biodiversity of potentially ochratoxigenic black aspergilli isolated from grapes. *International Journal of Food Microbiology* 132, 14-23, (2009).
31. BELLÍ N, MARÍN S, ARGILES E, RAMOS AJ, SANCHIS V, Effect of chemical treatments on ochratoxigenic fungi and common mycobiota of grapes (*Vitis vinifera). Journal of Food Protection,* 70, 157-163, (2007).