

Real-time PCR based procedures for detection and quantification of *Aspergillus carbonarius* in wine grapes

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Abstract

Aspergillus carbonarius is the main species responsible for ochratoxin A accumulation in wine grapes and consequently, its rapid and sensitive detection is increasingly investigated. A new real-time PCR (RTi-PCR) based procedure was developed for the rapid and specific detection and quantification of *A. carbonarius* in wine grapes. The procedure includes the use of the pulsifier equipment to remove conidia from grapes which prevents releasing of PCR inhibitors, and DNA extraction with the EZNA Fungal DNA kit. It reduced the time for *A. carbonarius* DNA extraction from grapes to 30 min. Two specific primers (AcKS10L/AcKS10R) delimiting a 161 bp fragment, and a probe were designed and directed to the β -ketosynthase domain of a polyketide synthase from *A. carbonarius*. Specificity was confirmed by testing primers towards purified DNA from 52 fungal strains, including reference and food isolates. Quantification was linear over at least 5 log units using both serial dilutions of purified DNA and calibrated conidial suspensions from *A. carbonarius*. The SYBR-Green I and TaqMan RTi-PCR approaches established were able to detect at least 2.4 and 24 genomic equivalents, respectively, using purified DNA. Results obtained from conidial suspensions, after DNA extraction, showed that at least 5 conidia per reaction should be present for a positive result with SYBR-Green I and 50 in the case of TaqMan. The quantification of fungal genomic DNA in artificially inoculated wine grapes performed successfully, with a minimum threshold of 10^3 conidia mL^{-1} for accurate quantification. The developed RTi-PCR assay is a promising tool in the prediction of potential ochratoxigenic risk, even in the case of low-level infections, and suitable for a rapid, automated and high throughput analysis.

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1. Introduction

Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, carcinogenic, immunotoxic, genotoxic and teratogenic effects, which has been associated with Balkan Endemic Nephropathy (Krogh, 1978; Kuiper-Goodman and Scott, 1989; Abouzied et al., 2002). OTA is receiving an increasing attention for its toxic effects and high incidence in a wide range of food commodities including grapes and related products such as wine and grape

juice (Battilani et al., 2006). In fact, in the European diet, wine and especially red wine, has been identified as the second major source of human exposure to OTA, following cereals (Anonymous, 1998). As a consequence, the European Commission has considered it is necessary to impose regulatory limits and has established 2 ppb as the maximum level of OTA in wine and grape products (Commission regulation No 123/2005 amending Regulation No 67 446/2001 as regards ochratoxin A) (Varga and Kozakiewicz, 2006).

OTA contamination in grapes takes place in the field and is caused by *Aspergillus* species belonging to section *Nigri* (Battilani and Pietri, 2002; Abarca et al., 2003; Belli et al., 2004; Martínez-Culebras and Ramón, 2007). Among these,

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Aspergillus carbonarius is considered the main responsible species for the OTA accumulation in grapes since it has been reported to have the highest ochratoxigenic potential (Cabañes et al., 2002; Battilani et al., 2003; Martínez-Culebras and Ramón, 2007). Fungi can be isolated from bunches starting from the early stages of the berry development even if their incidence is more relevant from early veraison (Battilani et al., 2003). The early detection of *A. carbonarius* is important to prevent OTA contamination in grapes and wine. For this reason, it is necessary to develop accurate methods which allow a rapid, sensitive and specific identification and quantification of *A. carbonarius* on grapes before harvesting.

Conventional culturing techniques are not only time-consuming but also are not able to give accurate results for fungal identification and quantification. Identification of black *Aspergillus* species based on morphological characters might be difficult and usually requires taxonomic expertise. In addition, spore isolation and enumeration may introduce a bias in favor of faster-growing species. Recent advances in DNA-based techniques such as real-time PCR (RTi-PCR) are providing new tools for fungal detection and quantification by detecting and quantifying their DNA. Like any other fungal quantification technique, RTi-PCR has also some drawbacks due to fungi might be present in vegetable samples in different forms i.e. spores, young mycelial fragments with many nuclei or old mycelial fragments with only a few nuclei. Nevertheless, it allows the specific detection of fungi in a mixed population which is by far the main advantage of PCR. In addition, post-PCR manipulations (such as gel electrophoresis) are not required, thus providing detection protocols that are highly sensitive, reliable and suitable for high throughput analysis (Schaad and Frederick, 2002).

RTi-PCR can be performed using different chemistries, such as SYBR® Green I dye (Witter et al., 1997) and TaqMan® (Livak et al., 1995). Both systems have proven useful in monitoring and quantification of fungal population in many food commodities such as meat (Bogs et al., 2006), coffee (Schmidt et al., 2004), and wheat (Geisen et al., 2004). Regarding *A. carbonarius*, the RTi-PCR reactions previously developed are a SYBR-Green I approach targeted to the acyltransferase domain of a polyketide synthase (PKS) (Atoui et al., 2007) and a TaqMan approach targeted to conserved regions of the *A. carbonarius* calmodulin gene (Mulè et al., 2006). Additionally, a positive correlation between OTA content and DNA quantity has been indicated for *Penicillium nordicum* and *Aspergillus ochraceus* using RTi-PCR (Geisen et al., 2004; Schmidt et al., 2004) and more recently, in *A. carbonarius* also (Mulè et al., 2006; Atoui et al., 2007). However, some studies found a non-linear correlation between toxin content and DNA quantity indicating that other factors are implicated in fungal toxin production and more studies are necessary prior to RTi-PCR can be proposed as a quick tool for fungal toxin detection (Sarlin et al., 2006; Schmidt et al., 2004). Currently, RTi-PCR quantification of *A. carbonarius* in grapes is a great extent the best alternative to conventional methods in order to investigate the relation between OTA producers and OTA content.

Nevertheless, problems sometimes occur with PCR mainly derived from inhibitors. They might interfere in one or more of the three essential points in the reaction, with the cell lysis necessary for extraction of DNA, by nucleic acid degradation or capture and also by inhibiting the polymerase activity for amplification of target DNA (Wilson, 1997). Organic compounds such as polyphenols and polysaccharides are among the inhibitors associated to food vegetables. Inhibition can manifest itself as complete reaction failure leading to false negative results or as reduced sensitivity of detection. The use of a convenient DNA extraction procedure would a great extent overcome inhibition of nucleic acid amplification. The RTi-PCR procedures previously developed for the quantification of *A. carbonarius* rely on the use of plant DNA extraction protocols. They are laborious extraction methods that provide high yields of DNA from plant tissues and still need a column purification step for a sensitive fungi detection (Mulè et al., 2006; Atoui et al., 2007). However, for the routine microbiological analysis of food, rapid and standardized extraction methods that allow a high sample throughput are desirable.

In the present study, the rapid, specific, sensitive and quantitative detection of *A. carbonarius* has been approached by designing new primers and probe, for SYBR-Green I and TaqMan RTi-PCR systems, in combination with a rapid and efficient fungi DNA extraction procedure tested in artificially inoculated wine grapes.

2. Materials and methods

2.1. Fungal strains and culture conditions

Black *Aspergillus* strains were isolated from Spanish vineyards and cocoa beans and are held in the Institute of Agrochemistry and Food Technology (IATA-CSIC). They were previously identified by ITS-RFLP profiles and tested for OTA production (Martínez-Culebras and Ramón, 2007). Reference strains were provided by Centralbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands) and the Spanish Type Culture Collection (CECT, Valencia, Spain) (see Table 1). They were all grown in Malt Extract Agar (MEA) plates at 25±1 °C for 5–7 days (Pitt and Hocking, 1997).

2.2. Fungal DNA extraction

Mycelia were collected from cultures growing on MEA plates, frozen in liquid nitrogen and ground to a fine powder. DNA extractions were performed using 100 mg of powder and the commercial EZNA Fungal DNA kit (Omega bio-teck, Doraville, USA) according to the manufacturer's instructions.

2.3. Sample preparation for sensitivity assays

Twenty five grams of wine grapes were homogenized in a 1:10 dilution of sterile 0.1% buffered peptone water (BPW, AES Laboratoire, Combourg, France) using either a pulsifier equipment (Microgen bioproducts, Surrey, UK) or a stomacher (Stomacher Lab-blender 400, Seward, London, UK) for

Table 1
Fungal strains used in this work for specificity assays

Species	Strain	Source of isolation	OTA	Presence of PCR product
<i>Aspergillus aculeatus</i> ^a	CECT 2968	Soil, India	–	–
<i>Aspergillus awamori</i> ^a	CECT 2907	Bran	+	–
<i>Aspergillus brasiliensis</i> ^a	CBS 101740	Soil, Brazil	+	–
<i>Aspergillus carbonarius</i> ^a	CBS 113.80	Cocoa, Nigeria	ND	+
	CBS111.26	Unknown substratum	ND	+
	C06-59	Cocoa, Sierra Leona	–	+
	C06-60	Cocoa, Ecuador	–	+
	C06-61	Cocoa, Ecuador	–	+
	C06-64	Cocoa, Ecuador	–	+
	C06-100	Cocoa, Ecuador	–	+
	W04-20	Wine grape, Spain	+	+
	W04-30	Wine grape, Spain	+	+
	W04-32	Wine grape, Spain	+	+
	W04-35	Wine grape, Spain	+	+
	W04-40	Wine grape, Spain	+	+
	W04-43	Wine grape, Spain	+	+
	W04-46	Wine grape, Spain	–	+
	W04-49	Wine grape, Spain	+	+
	W04-52	Wine grape, Spain	+	+
	W04-54	Wine grape, Spain	+	+
	W04-86	Wine grape, Spain	+	+
	W04-92	Wine grape, Spain	+	+
	W04-145	Wine grape, Spain	+	+
<i>Aspergillus costaricensis</i> ^a	CBS 115574	Soil, Costa Rica	–	–
<i>Aspergillus ellipticus</i>	CBS 707.79T	Soil, Costa Rica	–	–
<i>Aspergillus foetidus</i>	CBS114.49	Unknown substratum	–	–
	CBS 564.65	Unknown substratum, Japan	–	–
<i>Aspergillus heteromorphus</i>	CBS117.55	Trichophyton contaminated culture, Brazil	–	–
<i>Aspergillus japonicus</i> ^a	CBS114.51	Unknown substratum	–	–
<i>Aspergillus lacticofeatus</i>	CBS101883T	Coffee, Venezuela	+	–
<i>Aspergillus niger</i>	CECT 2088 ^a	Unknown substratum	ND	–
	CECT 2090	Unknown substratum	ND	–
	CECT 2091	Soil, Canada	ND	–
	W04-19	Wine grape, Spain	+	–
	C06-18	Cocoa, Guinea	–	–
	C06-19	Cocoa, Guinea	–	–
	C06-33	Cocoa, Sierra Leona	+	–
<i>Aspergillus ochraceus</i> ^a	CECT 2948	Unknown substratum	+	–
<i>Aspergillus piperis</i>	CBS 112811T	Grounded black pepper, Denmark	–	–
<i>Aspergillus pulverulentus</i>	CBS 558.65	Unknown substratum	ND	–
<i>Aspergillus tamari</i>	W04-15	Wine grape, Spain	–	–
<i>Aspergillus tubingensis</i>	CBS 136.52	Kuro-koji, Japan	–	–
	CBS134.48 ^a	Unknown substratum	ND	–
	C06-2	Cocoa, Sierra Leona	–	–
	C06-24	Cocoa, Sierra Leona	+	–
	W04-38	Wine grape, Spain	ND	–
	W04-53	Wine grape, Spain	ND	–
	W04-57	Wine grape, Spain	ND	–
<i>Aspergillus usamii</i>	CBS 101700	Unknown substratum	+	–
<i>Botrytis cinerea</i> ^a	W04-186	Wine grape, Spain	–	–
<i>Cladosporium</i> spp. ^a	W04-122	Wine grape, Spain	–	–
<i>Fusarium</i> spp. ^a	W04-101	Wine grape, Spain	–	–
<i>Penicillium</i> spp. ^a	W04-28	Wine grape, Spain	–	–

ND: OTA production was not determined. W04 and C06: Strains isolated from Spanish vineyards and cocoa beans, respectively. CBS: Centralbureau voor Schimmelcultures; CECT: Spanish Type Culture Collection.

^a Strains tested by SYBR-Green I and TaqMan RTi-PCR.

comparative purposes. Aliquots were taken from each bag, serially diluted in 0.1% BPW, and plated on dichloran rose bengal chloramphenicol agar (DRBC, Oxoid, Basingstoke, Hampshire, UK). Molds were enumerated after incubation at 25 ± 1 °C for 5–7 days. One milliliter aliquots of pulsed wine

grape samples were tested in triplicate by RTi-PCR using the *A. carbonarius* specific primers AcKS10L/AcKS10R and amplification conditions described below (Section 2.6.). Samples that tested negative for *A. carbonarius* by culture techniques and RTi-PCR were used for the inoculation experiment.

For grape inoculation, fungal conidia of *A. carbonarius* W04-40 were prepared flooding 3 plates (6 days old) of MEA with 5 mL of sterile nanopure water containing 0.05% Tween 80 (Fluka Biochemika, Steinheim, Germany), and rubbing surface with a glass rod. The conidial suspension was filtered through Whatman paper No 1, diluted in sterile nanopure water, as necessary, and quantified by plate count on MEA, and by microscopy, using a Neubauer counting chamber. Aliquots of pulsed wine grape samples (0.9 mL), previously tested as negative for *A. carbonarius* by PCR, were inoculated with 0.1 mL of 10^3 , 10^4 , 10^5 and 10^6 conidia mL^{-1} , respectively. One milliliter non-inoculated aliquot was included as negative control.

For DNA extraction, 1 mL aliquots of both artificially inoculated wine grape samples and pure conidia of *A. carbonarius* W04-40 at different concentrations (10 to 10^6 conidia mL^{-1}) were centrifuged for 3 min at 13,000 *g*. Pellets were washed in 0.5 mL of TE (10 mmol L^{-1} Tris-HCl; 1 mmol L^{-1} EDTA, pH 8), centrifuged at 13,000 *g* for 3 min and resuspended in 50 μL of sterile nanopure water. They were boiled (95 °C for 10 min) to breakdown conidia and release the DNA. Samples were cooled on ice for 10 min. Finally, DNA isolation was performed by EZNA Fungal DNA kit (Omega bio-teck, Doraville, USA), starting from step 1 of protocol B where 600 μL of buffer FG1 were added to 50 μL of pellets resuspended in water. In the final step, DNA was eluted in 100 μL of sterile nanopure water and kept at -20 °C until used as template for PCR amplification.

2.4. Primer and TaqMan probe design

Primers and TaqMan probe reported in Table 2 were derived from a conserved region in the β -ketosynthase (KS) domain of a PKS gene (AcKS10) from *A. carbonarius* (Genbank accession number: AY540952). The design of the primers and TaqMan probe for RTi-PCR assays was carried out using the Primer Express, version 2.0 software (Applied Biosystems Division, Perkin-Elmer Co., Foster city, CA, USA). Primers delimit a 161 bp fragment. TaqMan probe was labelled at the 5' and 3' ends with a 6-carboxy-fluorescein group (FAM) and 6-carboxy-tetramethyl-rhodamine (TAMRA), respectively. None of them showed homology with any bacterial or fungal gene different to *A. carbonarius* when tested using the BLAST tool in the public

database (www.ncbi.nlm.nih.gov/BLAST/). Primer and probe synthesis were ordered from ABI PRISM Primers and TaqMan Probe Synthesis Service (Applied Biosystems, Madrid, Spain).

2.5. Conventional PCR amplification conditions

PCR reactions using the *A. carbonarius* specific primers AcKS10L/AcKS10R and the universal primers its5/its4 (White et al., 1990) were performed in 50 μL as final volume, containing 200 ng of DNA, 50 mM KCl, 10 mM Tris-HCl, 100 μM (each) dNTP, 1 μM of each primer, 2 mM MgCl_2 and 1 U of DNA polymerase (Netzyme, Molecular Netline Bioproducts, N.E.E.D, SL, Valencia, Spain).

Amplification conditions were: 35 cycles consisting of 1 min at 95 °C, 1 min at 52 °C and 1 min at 72 °C. The reaction mixtures were incubated in a thermocycler (Techne TC-512). Fifteen microliters of PCR products were electrophoresed through 2% agarose (Pronadisa, Hispanlab, Madrid, Spain) gel in TAE buffer [40 mM Tris-acetate, pH 7.6 and 1 mM Na acetic acid (EDTA)]. After electrophoresis, gels were stained with ethidium bromide (0.5 mg mL^{-1}), and the DNA bands visualized under UV light. Sizes were estimated by comparison with a DNA standard length (GeneRuler TM 100 pb DNA ladder, MBI Fermentans, Vilnius, Lithuania).

2.6. RTi-PCR amplification conditions

RTi-PCR reactions, with SYBR-Green I and TaqMan probe, were performed using the SYBR-Green I Core Reagents and the TaqMan Core Reagents, respectively (both from Applied Biosystems, Madrid, Spain). Amplification mixtures for RTi-PCR reactions with SYBR-Green I, contained in a final volume of 20 μL , 1 \times PCR Buffer, 200 μM each dATP, dCTP, dGTP and 400 μM dUTP; 1 U of Amperase uracil *N*-glycosidase; 2 mM MgCl_2 ; 0.9 U of AmpliTaq Gold DNA polymerase and 5 μL of template DNA (10 ng for specificity assay). Different concentrations of each primer (100, 200 and 300 nM) were assayed. Amplification mixtures for RTi-PCR reactions with TaqMan probe, contained in a final volume of 20 μL , 1 \times TaqMan Buffer A, 200 μM each dATP, dCTP, dGTP and 400 μM dUTP; 1 U of Amperase uracil *N*-glycosidase; 2.5 mM MgCl_2 ; 0.9 U of AmpliTaq Gold DNA polymerase and 5 μL of template DNA. Different concentrations of TaqMan probe (50, 100, 150 and 200 nM) were assayed. RTi-PCR assays, using either SYBR-Green I or TaqMan probe for fluorescence emission, were carried out in a GeneAmp® 5700 Sequence Detection System (PE Biosystems, Foster city, CA, USA) programmed to hold at 50 °C for 2 min, to hold at 95 °C for 10 min, and to complete 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Reactions were done in triplicate. PCR results were given as the increase in the fluorescence signal of the reporter dye detected and visualized by the GeneAmp 5700 SDS software provided with the GeneAmp® 5700 SDS (Applied Biosystems). Threshold cycle (C_T) values represent the PCR cycle in which an increase in fluorescence, over a defined threshold, first occurred, for each amplification plot.

Table 2
Aspergillus carbonarius specific primers and TaqMan probe set

Primer	Size	Oligonucleotide sequence	Tm	Location within gene ^a
AcKS10R	22	5'-CCC TGA TCC TCG TAT GAT AGC G-3'	65.1	362 to 340
AcKS10L	22	5'-CCG GCC TTA GAT TTC TCT CAC C-3'	65.4	201-223
Probe ^b	23	5'-FAM-AGA ACG CTG ATG GGT ATG CGC GG-TAMRA-3'	70.0	275 to 252

^a β -Ketosynthase domain of a polyketide synthase gene (Genbank accession number: AY540952).

^b TaqMan probe labelled at the 5' end with 6-carboxy-fluorescein (FAM), and at the 3' end with 6-carboxy-tetramethyl-rhodamine (TAMRA).

2.7. Melting-curve analysis

For RTi-PCR with SYBR-Green I, melting curves were programmed in order to check the expected amplification product. The thermal protocol for dissociation is defined as 15 s at 95 °C, 20 s at 60 °C and 20 min slow ramp between 60 and 95 °C, after the RTi-PCR program. The data for dissociation curve is captured during this slow ramp. The melting curve was visualized with the software in the dissociation Window using the corresponding GeneAmp 5700 SDS software procedure (Applied Biosystems).

2.8. Quantification assays

Standard curves were calculated for quantification purposes using: (i) ten-fold serial dilutions of purified DNA from *A. carbonarius* W04-40 in sterile nanopure water covering the range from 10 to 1×10^{-6} ng per reaction. DNA concentration was determined fluorometrically using the Fluorescent DNA quantitative kit (Bio-Rad, California, USA) and a VersaFluor® Spectrofluorimeter (Bio-Rad, London, England); (ii) DNA extracted from ten-fold serial dilutions of *A. carbonarius* W04-40 conidial suspensions in sterile saline (0.8% NaCl), covering the range from 1 to 1×10^7 conidia mL⁻¹. PCR amplification reactions were carried out in triplicate in 3 independent experiments. Standard curves were generated by plotting the genomic DNA and conidium suspensions from *A. carbonarius* W04-40 against the C_T values exported from the GeneAmp® 5700 Sequence Detection System (PE Biosystems, Foster city, CA, USA) for each plate. The C_T values for unknown samples were extrapolated from standard curves.

2.9. Detection limits of RTi-PCR for *A. carbonarius* in artificially inoculated wine grapes

Sensitivity assays were carried out on artificially inoculated wine grapes as indicated above using BPW. Aliquots of pulsed grape samples (0.9 mL) were inoculated with 0.1 mL of 10^3 , 10^4 , 10^5 , 10^6 conidia mL⁻¹ (determined by plate count in MEA). After DNA isolation and elution in 100 µL

of sterile nanopure water, 5 µL of DNA solution was used as template for RTi-PCR amplification. Reactions were carried out in triplicate in 3 independent experiments. In order to assess the absence of PCR inhibitors in the food matrix, amplification control was conducted in triplicate using as template, 5 µL DNA from the non-inoculated grape homogenate aliquot and 2 ng *A. carbonarius* purified DNA.

3. Results

3.1. Establishment of the DNA extraction procedure from inoculated wine grape samples

Mold counts obtained from natural contaminated grapes after homogenization in 0.1% BPW using the pulsifier equipment and the stomacher were 2.64 ± 0.20 and 2.65 ± 0.25 log CFU g⁻¹, respectively. Thus, the efficacy of pulsifier and stomacher equipments removing molds from grape samples were almost the same. Pulsifier was selected for further food sample analyses since it prevents food matrix breakdown avoiding the release of potential PCR inhibitors. The combination of pulsifier and EZNA Fungal DNA kit to isolate DNA from inoculated grapes, allowed completing the DNA extraction process in 30 min.

3.2. Primer and probe specificity tests

Primer specificity was first evaluated by conventional PCR using as template, DNA from a large number of reference strains and food isolates (Table 1). They include *A. carbonarius*, most of the black *Aspergillus* species currently recognized, as well as other fungal species commonly found on grapes (*Botrytis cinerea*, *Cladosporium* spp., *Fusarium* spp., *Penicillium* spp.). The specific 161 bp was only obtained in the *A. carbonarius* reference strains and food isolates, confirmed as members of the species (Table 1). Amplification of the 5.8S-ITS region using universal primers was obtained in all strains, thus confirming the DNA suitability when negative amplification results were obtained using the primers AcKS10L and AcKS10R.

Afterwards, primer and probe specificity was tested by RTi-PCR using the optimized reaction conditions for SYBR-Green I

Table 3
 C_T values corresponding to standard curves obtained using SYBR-Green I and TaqMan systems, with genomic DNA and conidial suspensions from *Aspergillus carbonarius* W04-40

Pure DNA of conidia			Conidium suspensions		
Genome equivalents ^a	SYBR-Green mean $C_T \pm SD$	TaqMan mean $C_T \pm SD$	Conidia/reaction	SYBR-Green I mean $C_T \pm SD$	TaqMan mean $C_T \pm SD$
2.4×10^5	23.76 ± 0.70^b	29.96 ± 0.20^b	5×10^5	24.27 ± 0.77	30.45 ± 0.61
4.4×10^4	25.01 ± 0.63				
2.4×10^4	27.20 ± 0.10	32.72 ± 0.16	5×10^4	27.05 ± 1.00	33.06 ± 0.27
4.4×10^3	28.49 ± 0.39				
2.4×10^3	30.05 ± 0.46	35.70 ± 0.39	5×10^3	30.61 ± 1.03	35.83 ± 0.92
4.4×10^2	31.99 ± 0.77				
2.4×10^2	32.85 ± 0.22	38.08 ± 0.43	5×10^2	33.12 ± 0.78	38.18 ± 0.99
44	35.53 ± 0.10				
24	35.66 ± 0.73	39.75 ± 0.43	50	35.12 ± 0.44	39.97 ± 0.10
2.4	38.05 ± 0.13	40.00 ± 0.00	5	37.05 ± 0.10	40.00 ± 0.00

^a Genomic DNA weight of *A. carbonarius* was calculated assuming it to be similar to that of *A. niger* (4.13×10^{-5} ng, for a haploid genome).

^b Data represent the mean \pm standard deviation (SD) of the 3 independent experiments, each consisting of triplicate samples.

and TaqMan (100 nM primers and 100 nM probe) and 20 ng of purified DNA from 20 *A. carbonarius* strains and 12 other fungal species (Table 1). The C_T values recorded from *A. carbonarius* were in the range of 23.5 ± 1.3 with SYBR-Green I and 30 ± 1.2 with TaqMan while no DNA amplification ($C_T=40$) was detected in strains from the other species tested (Table 1). These results indicate that both RTi-PCR systems are specific and suitable for the *A. carbonarius* detection. In addition, the amplicons obtained for *A. carbonarius* strains had T_M values that were in the range of $83.1\text{--}83.7$ °C which confirmed the homogeneity of the targets selected in this species.

3.3. Standard curves and detection limits of the RTi-PCR using SYBR-Green I and TaqMan systems

Table 3 summarizes the mean C_T values of three replicates obtained with SYBR-Green I and TaqMan systems in three

independent experiments. They correspond to: i) ten-fold dilutions of genomic DNA isolated from *A. carbonarius* W04-40 in the range of 2.4 to 2.4×10^5 genome equivalents per reaction, and ii) DNA extracted from calibrated conidial suspensions of the same strain in a range of 5 to 5×10^5 conidia per reaction. Representative amplification plots corresponding to standard quantification curves are shown in Fig. 1. They showed a linear correlation between \log_{10} input DNA and C_T with slope and R^2 (square regression coefficient after the linear regression) values very similar in three independent assays. The slopes of the linear regression curves, calculated using genomic DNA were -2.99 with SYBR-Green I and -2.56 with TaqMan, and R^2 values were above 0.99 indicating that the RTi-PCR systems were highly linear (Fig. 1A, C). Application of this method to calibrated conidial suspensions (conidia mL^{-1}) provided a positive correlation between conidia and haploid genome weight. Genomic DNA weight of *A. carbonarius* was assumed to be similar to that of *A. niger* (4.13×10^{-5} ng, for a

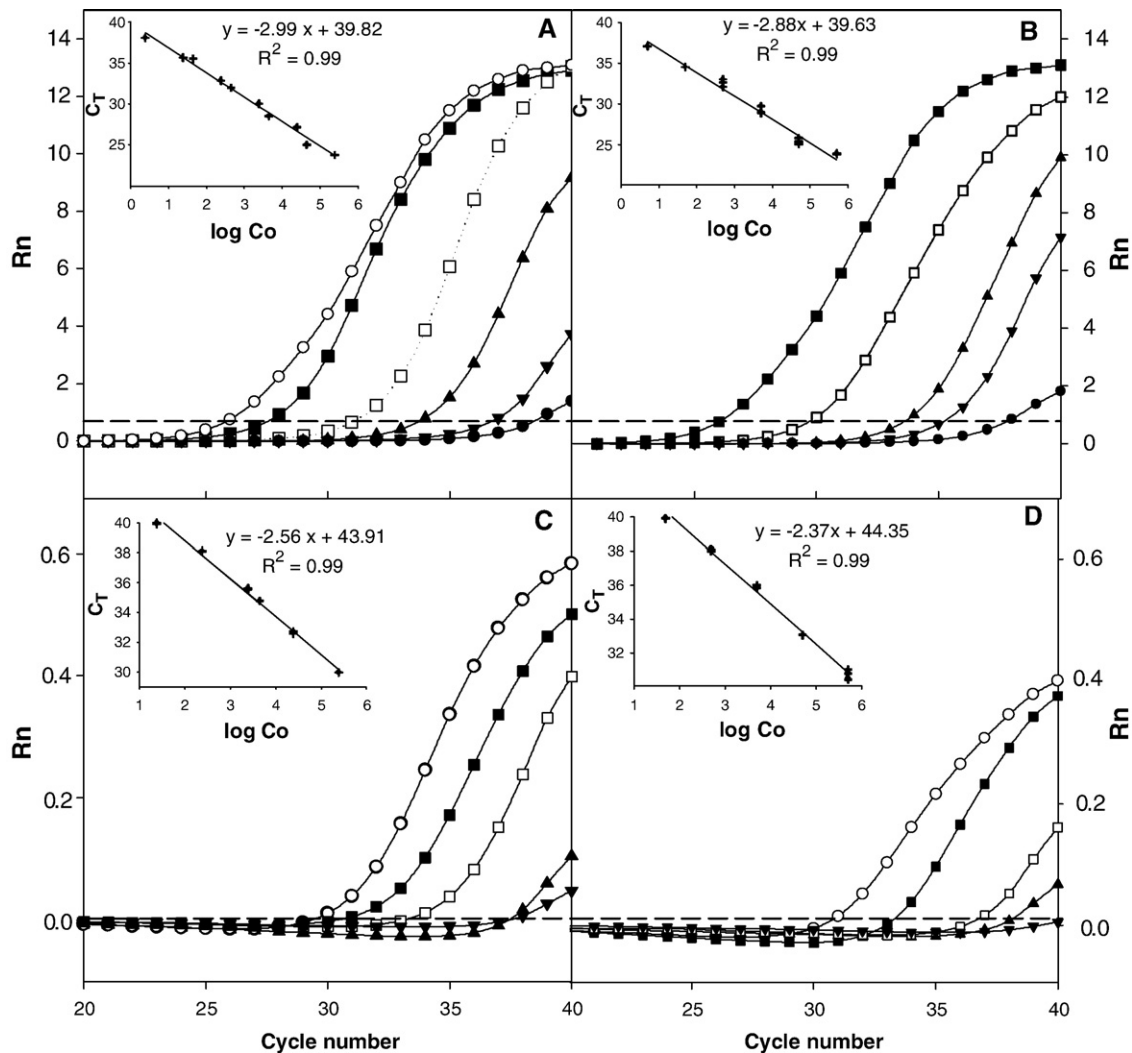


Fig. 1. Representative amplification plots corresponding to standard curves obtained from genomic DNA serial dilutions (A, C) and calibrated conidial suspensions (B, D) of *A. carbonarius* W04-40 with SYBR-Green I (A, B) and TaqMan (C, D). Insets show representative standard curves generated from the amplification data: C_T values are plotted against the conidia concentration (C_0) as \log_{10} of genome equivalents/reaction (4.13×10^{-5} ng, for haploid genome) (A, C) and \log_{10} of conidia/reaction (B, D) determined by plate count. (A, C): \circ , 2.4×10^5 ; \blacksquare , 2.4×10^4 ; \square , 2.4×10^3 ; \blacktriangle , 2.4×10^2 ; \blacktriangledown , 24; \bullet , 2.4 target molecules per reaction. (B, D): \circ , 5×10^5 ; \blacksquare , 5×10^4 ; \square , 5×10^3 ; \blacktriangle , 5×10^2 ; \blacktriangledown , 50; \bullet , 5 conidia per reaction.

haploid genome) reported in the website <http://www.broad.mit.edu/annotation/fungi/fgi/history.html>. Linear regression analysis of C_T values corresponding to calibrated conidial suspensions yielded R^2 values above 0.99 and the slopes were -2.88 with SYBR-Green I and -2.37 with TaqMan (Fig. 1B, D). These results indicate that both RTi-PCR systems can be used to quantify *A. carbonarius* conidia.

Regarding sensitivity of RTi-PCR systems, 2.4 genome equivalents were detected with SYBR-Green I meanwhile 24 were needed for a positive reaction using the TaqMan mode (Table 3). Results obtained using DNA extracted from conidial suspensions, showed at least 5 and 50 conidia in the reaction should be present for a positive reaction with SYBR-Green I and TaqMan, respectively (Table 3). Differences between both approaches can be explained by DNA losses during DNA extraction but nevertheless they kept in the same log order.

3.4. Sensitivity of the RTi-PCR assay on artificially inoculated wine grapes

C_T values obtained from DNA extracted using artificially inoculated grapes were analyzed in order to evaluate the extent to which the specific RTi-PCR procedure is applicable to *A. carbonarius* detection in wine grapes. Results of three replicates are shown in Table 4. A linear correlation was observed between RTi-PCR quantification (C_T values) and plate counts in the range from 5 to 5×10^3 conidia per reaction. The slopes of the linear regression analysis corresponding to these dilutions were -3.25 with SYBR-Green I and -2.10 with TaqMan, and R^2 values were above 0.99. These values indicated that linearity of the amplification reaction was also kept in the inoculated matrix. The linear correlation produced in this case with all three assays was comparable in threshold and dynamic range to those represented in Fig. 1. Therefore, it corroborated the efficiency of the DNA extraction method from grapes since it did not interfere with RTi-PCR assays. According to the mean C_T values obtained from a total of 9 replicates in three independent amplification experiments, 66.6% of positive reactions were recorded with TaqMan in samples containing 50 conidia per reaction (Table 4). If a 100% of positive reactions are considered, the limits of detection were 5 and 500 conidia with SYBR-Green I and TaqMan, respectively. Thus, the SYBR-Green I and TaqMan RTi-PCR systems proved efficient for detection and quantification of *A. carbonarius* in grapes. Values derived from

standard curves of calibrated conidial suspension yielded more accurate quantification than the ones derived from DNA standard curves. Nevertheless, in both cases the quantification values remained in the same log order indicating that any of them could be useful for quantification analyses (Table 4).

4. Discussion

Recently, much interest has been focused on detection and quantification of *A. carbonarius* which is predominantly responsible for the contamination of grapes and wine with OTA (Battilani and Pietri, 2002; Cabañes et al., 2002). In the present study, a rapid, specific and sensitive RTi-PCR assay for the detection and quantification of *A. carbonarius* on grapes was developed. To achieve this, a very rapid procedure for DNA extraction directly from grape berries was used; two primers and a probe were designed; primer specificity was examined using a large number of fungal strains, and both SYBR-Green I dye and TaqMan detection chemistries were evaluated.

Regarding fungal DNA isolation, crude extracts from grapes contain many PCR-inhibiting substances, such as tannins, polysaccharides and pigments that can lead to poor PCR amplification. Previous RTi-PCR assays for detection and quantification of *A. carbonarius* on grapes (Mulè et al., 2006; Atoui et al., 2007) used a combination of several plant DNA extraction techniques (Steenkamp et al., 1994; Kim et al., 1997; Loureiro et al., 1998). This protocol is not only time-consuming but also needed purification step by spin columns to overcome problems of sensitivity in the RTi-PCR experiments. In the present study, fungal DNA extraction from grapes was approached combining the use of pulsifier equipment for sample homogenization and a commercial kit for DNA purification. Instead of crushing the food sample, we introduced the use of the pulsifier equipment which beats the sample bag very rapidly using an oscillating metal ring (Fung et al., 1998). In this way, conidia are removed from samples but there is minimal breakdown of the grapes, which prevents releasing of PCR inhibitors. Furthermore, DNA extraction method developed by using pulsified samples followed by EZNA Fungal DNA kit was very rapid and allowed to complete RTi-PCR assays in 2 h. Thus, these procedures considerably reduce the time needed for *A. carbonarius* detection in grapes and coffee by other authors (Mulè et al., 2006; Sartori et al., 2006; Atoui et al., 2007).

Table 4
Quantification of *A. carbonarius* in artificially inoculated grapes using DNA and conidial suspension standard curves

Inoculated conidia (conidia mL ⁻¹)	SYBR-Green I		TaqMan			
	Positive signals/total reactions	Quantification from standard curves (conidia mL ⁻¹) ^a		Positive signals/total reactions	Quantification from standard curves (conidia mL ⁻¹) ^a	
		DNA	Conidia		DNA	Conidia
1×10^5	9/9	$4.8 (0.3) \times 10^4$	$9.9 (0.1) \times 10^4$	9/9	$3.7 (0.3) \times 10^4$	$1.0 (0.2) \times 10^5$
1×10^4	9/9	$4.5 (0.4) \times 10^3$	$9.7 (0.2) \times 10^3$	9/9	$3.5 (0.3) \times 10^3$	$9.2 (0.3) \times 10^3$
1×10^3	9/9	$2.4 (0.2) \times 10^2$	$3.0 (0.1) \times 10^2$	6/9	$8.8 (0.2) \times 10^2$	$1.0 (0.2) \times 10^3$
1×10^2 ^b	9/9	$3.0 (0.1) \times 10$	$3.0 (0.1) \times 10$	0/9	–	–

^a Data represent the mean of the 3 independent experiments, each consisting of triplicate samples. Values in brackets represent standard deviation at $P \leq 0.05$.

^b It corresponds to 5 conidia per reaction.

For primers and probe design, we used the KS domain of a PKS sequence from *A. carbonarius* (AcKS10), as target for PCR detection. To detect and quantify mycotoxin producing fungi, the best target would be genes involved in the mycotoxin biosynthesis pathway. Recently, the KS domain sequence from *A. carbonarius* (AcKS10) was identified (Atoui et al., 2006). It displayed about 60% identity to AoLC35-12 sequence, which was found to join a sequence encoding for acyltransferase (AT) of a PKS involved in OTA biosynthesis in *A. ochraceus* (O'Callaghan et al., 2003). The primers designed (AcKS10L/AcKS10R) proved to be highly specific when tested by conventional and RTi-PCR in a very large number of fungal strains yielding amplification only in *A. carbonarius* strains.

Following optimization of the reaction conditions, the SYBR-Green I and TaqMan RTi-PCR developed were able to detect at least 2.4 and 24 genomic equivalents, respectively, using purified DNA. These results are in the same range as the detection limit reported for the TaqMan RTi-PCR system developed by Mulè et al. (2006), and about 1 log order lower (more sensitive) than the detection limits reported for the SYBR-Green I system used by Atoui et al. (2007). Results obtained from conidial suspensions, after DNA extraction, showed at least 5 conidia per reaction should be present for a positive result with SYBR-Green I, and 50 conidia in the case of the TaqMan system. Comparison with previous studies on RTi-PCR detection of *A. carbonarius* (Mulè et al., 2006; Atoui et al., 2007) was not possible since they tested sensitivity using only purified DNA. However, our results are in the same range as the detection limit reported for other fungi such as *B. cinerea* (10 conidia) (Suárez et al., 2005). The assay proved highly reproducible, with low variation in the C_T values obtained in three independent experiments.

In order to evaluate the suitability of these RTi-PCR systems for *A. carbonarius* detection in grapes we applied them to artificially inoculated samples. Results demonstrated that around 5×10^2 and 5×10^3 conidia g^{-1} have to be present on grapes to get a positive result with SYBR-Green I and TaqMan, respectively. These detection limits correspond to 5 and 50 conidia per reaction, respectively, the same detection limit as obtained from conidial suspensions. Thus, despite the DNA potential losses derived from DNA extraction process, the RTi-PCR developed proved equally sensitive and allowed the specific detection of *A. carbonarius* in the presence of the microbiota naturally occurring in wine grapes. With regards to food safety, Atoui et al. (2007) established that *A. carbonarius* DNA content has to be lower than 10 ng DNA g^{-1} grape berry (4.1×10^6 conidia g^{-1} grape) to fulfill with the maximum OTA permitted levels in the European Union (Commission regulation No 123/2005 amending Regulation No 67 446/2001 as regards ochratoxin A). However, a significantly more sensitive assay is particularly important for the study of low-level infections in order to prevent OTA contamination in grapes and wine.

On the basis of these results, we conclude that the SYBR-Green I and TaqMan assays developed in this study proved to be specific and sensitive, thus providing a fast and accurate tool for detection and quantification of *A. carbonarius* in wine grapes.

In addition, the SYBR-Green I may be economically more convenient than the TaqMan system for routine food analyses, since it does not require additional cost for the probe. Currently, the guideline proposed by the European Standardization Committee (CEN) in collaboration with the International Standard Organization (ISO) on the application of PCR for the detection of food-borne pathogens (Anonymous, 2002) requires an internal amplification control (IAC) in order to prevent false negative results derived from the presence of PCR inhibitors. Besides there are several approaches for the construction of an IAC, it has to be co-amplified with the target DNA in a multiplex PCR fashion which might affect the sensitivity of detection by competing reasons. We better propose the use of parallel amplification controls by the inclusion of purified DNA in some of the testing DNA extracts. It would a great extent serve as a positive amplification control without interfering in sensitivity. The application of this RTi-PCR assay would contribute to food safety improving the prediction of the main source of OTA contamination in wine grapes, thereby reducing the risk of OTA in wine.

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