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RESEARCH ARTICLE

Early identification of *Aspergillus carbonarius* in artificially and naturally contaminated grape berries by real-time polymerase chain reaction

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Abstract

Objectives The contamination of agricultural commodities with mycotoxins can occur without visible fungal contamination. Therefore, it is very important to develop a method for detecting very low levels of *Aspergillus carbonarius* DNA, which is the main agent responsible for ochratoxin A contamination in grape and wine. *Methods* The aim of this study was to detect the presence of *A. carbonarius* in grapes early during different stages of fungal development by the SYBR® Green real-time polymerase chain reaction or quantitative polymerase chain reaction approach by designing species-specific primers (Ac*pks*) on the basis of the polyketide synthase sequences. *Results* The real-time polymerase chain reaction amplification results show early detection of a specific *A. carbonarius* from non-symptomatic grape berries harvested directly from the field. Moreover, the amplification of fungal DNA extracted from grape berries artificially inoculated with *A. carbonarius* has shown an amplification starting from 6 h after inoculation, i.e. when the mycelium is not yet visible under stereomicroscope ($\times 100$) observation. *Conclusion* The results indicate that these highly specific and sensitive polymerase chain reaction-based methods are able to discriminate grape berries infected with *A. carbonarius* from uninfected ones. Thus, in a working day, it is possible to harvest the berries, extract high-quality DNA and perform *A. carbonarius*-specific real-time polymerase chain reaction amplification. The method proposed in this work could contribute toward food safety (e.g., wine and table grapes) because it could be used to predict the potential risk of contamination by potentially ochratoxigenic strains of *A. carbonarius*.

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Introduction

Aspergillus carbonarius is an ochratoxin A (OTA)-producing fungus and is the main agent responsible for the toxin contamination of grapes and wine (Serra *et al*., 2003). OTA is a secondary metabolite, produced by fungi belonging to the *Aspergillus* and *Penicillium* genera, and is nephrotoxic, nephrocarcinogenic, teratogenic and immune-suppressive in animals and humans (Stoev, 1998). OTA was discovered in 1965 as a fungal metabolite toxic to animals (Van der Merwe *et al*., 1965). The International Agency for Research on Cancer classified OTA as a group B carcinogen (IARC, 1993), that is, a possible carcinogen to humans. OTA has been detected in food products such as wine, beer, grape juice, dried fruit, meat, figs, coffee and cereals (Abarca *et al*.,

1994; Logrieco *et al.*, 2003; Serra *et al.*, 2003; Stefanaky *et al.*, 2003; Taniwaki *et al.*, 2003).

As a consequence, the European Commission has imposed regulatory limits and has established $2\,ng\,g^{-1}$ as the maximum level of OTA accepted in wine and grape products (Commission Regulation 1881/2006). *A. carbonarius* and black Aspergilli in general (*Aspergillus niger*, *Aspergillus tubingensis*) are known to be 'potential producers' of OTA; between these, *A. carbonarius* is the most dangerous as most of its strains (90–100%) display the highest ochratoxigenic potential (Horie, 1995; Téren *et al.*, 1996; Heenan *et al.*, 1998; Cabañes *et al.*, 2002; Serra *et al.*, 2003; Medina *et al.*, 2005; Perrone *et al.*, 2006). The exact identification of these species is very important in avoiding the overestimation of toxicological contamination and related risks.

The identification of fungi solely based on morphological features might be difficult, time consuming and usually requires taxonomic expertise. Because of this, it is necessary to develop methods that allow specific, sensitive and rapid identification and quantification of *A. carbonarius* on grapes before harvesting. Recent advances in DNA-based techniques such as real-time polymerase chain reaction (PCR) are providing new tools for fungal detection and quantification in complex matrices. The PCR procedures developed previously for the identification of *A. carbonarius* rely on the use of plant DNA extraction protocols. These 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).

Real-time PCR assays play an important role, among molecular screening methods, because of the accelerated diagnostic outcome. The system has proven useful in monitoring and quantifying fungal populations in several food commodities (Geisen *et al.*, 2004; Morello *et al.*, 2007), such as grapes, to correlate *A. carbonarius* DNA with OTA content (Mulè *et al.*, 2006; Atoui *et al.*, 2007) and to assess the suitability of SYBR® Green I versus TaqMan in RTi-PCR systems (Selma *et al.*, 2008; González-Salgado *et al.*, 2009).

In the present study, we have developed an SYBR® Green real-time PCR assay for the early detection *A. carbonarius* strains in artificially inoculated and naturally contaminated grape berries of some varieties (endemic and not) used in Italy for producing wine of high quality. The quantitative detection of *A. carbonarius* DNA has been approached by designing a primer set based on a polyketide synthase gene sequence (Ac*KS10*) probably involved in OTA biosynthesis (O'Callaghan *et al.*, 2003).

Materials and methods

Isolation of natural fungal contaminants from grape berries

Several grape berries (growth stage: ripe for harvest), derived from *Vitis vinifera* var. Bombino nero, Cesanese di Affile, Malvasia, Merlot, Montepulciano, Nero d'Avola; Refosco faedis, Refosco vescovo (Velletri, Lazio, Italy), Dama, Sangiovese, Negroamaro (Bari, Puglia, Italy), were homogenized using a Waring blender in which 1 g of the sample was suspended in distilled and sterilized water by stirring for 1 h. A conidial suspension (0.1 ml) was plated in a Potato Dextrose Agar (PDA) medium in the presence of streptomycin (300 p.p.m.) and neomycin (150 p.p.m.), to inhibit the growth of bacteria, and incubated at 30 °C. After 2 days of incubation, each growing monoconidial fungal colony was transferred to Czapek Yeast Agar (CDA). These colonies were incubated at 30 °C for 7–10 days in the dark. The purity of these colonies was tested by three additional single-spore transfers on CDA medium. The different fungi were identified by a morphological tool.

Artificial contamination of grape berries

A conidial suspension (1–5 conidia/10 μL) of the *A. carbonarius* strain M (item not yet assigned in the fungi collection ISPA-CNR, Institute of Sciences of Food Production) was used for infecting berries (ripe for harvest) of 1 g each (*V. vinifera* var. Merlot) superficially sterilized by Clorex (NaClO) 2% solution. The exact number of conidia to be inoculated was obtained using a counting chamber (Thoma) and carrying out serial dilutions in order to achieve the desired concentration (1–5 conidia/10 μL). The same conidial suspension (10 μL) was plated in the PDA medium in order to test conidia vitality. Inoculation was performed by pipetting a small volume (10 μL – to avoid dripping) onto the upper surface of the berries, contained in sterile Petri plates. Non-inoculated and superficially sterilized berries were used as a negative control. The berries were incubated for 6, 12, 24, 40, 48, 72, 90 and 120 h under fixed conditions (30 °C). After incubation, each berry was analyzed using a stereo microscope, at each time interval considered, to check the presence of *A. carbonarius*. The berries were flushed in a known volume of a solution of sterile water and Triton 0.01% v/v. The conidia present in the washing water were measured using a counting chamber (Thoma).

DNA extraction from grape berries

An amount of fresh grapes berries were ground in liquid nitrogen and an aliquot (300 mg) was weighed and incubated with extraction buffer (Tris-HCl 200 mM pH 8.5, NaCl 250 mM, EDTA 25 mM, SDS 0.5%) for 60 min at 65 °C in an orbital shaker. After incubation, samples were put on ice for 10 min and centrifuged at 12 000 r.p.m. for 15 min at 4 °C. The supernatant was collected in a 2 mL tube and 3/10 volume of sodium acetate 4 M was added. This solution
was placed on ice for 30 min and centrifuged at 12 000 r.p.m. for 10 min at 4 °C and the supernatant was transferred, extracted with phenol–chloroform–isoamylic alcohol (25:24:1) and precipitated by adding 0.5 volume of cold 2-propanol. The same procedure (except for the sodium acetate phase) was performed for extracting DNA from 30 mg of lyophilized mycelium of the different fungi.

Real-time PCR operative conditions (optimized for SYBR® Green)

A. carbonarius-specific primers Acpksfor1/Acpksrev2 were derived from a conserved region in the β-ketosynthase (KS) domain of a PKS gene from *A. carbonarius* (Accession number: AY540952). This sequence was chosen among a set of five *pks* genes present in the *A. carbonarius* genome. In particular, the primers were designed for specific regions inside the KS domain of the Ac*KS10* gene sequence, which shares up to 60% of identity with Ao*LC35-12*, a PKS of *A. ochraceus* involved in OTA biosynthesis (O'Callaghan *et al.*, 2003). The primer pair Acpksfor1/Acpksrev2 (FOR 5′-TCACCTTTTGCGCACGGCTA-3′; REV 5′- GCGTCGTA CAAAGCCTCTT -3′) was designed using the Primer Express 3.0 software (fragment amplification of 255 bp length). Real-time PCR was carried out with triplicates of 20 µL reaction mixture, which contains 10 µL of SYBR® Green I Mix 1 × (Quantace), 0.8 µL of each primer Acpksfor/Acpksrev2 (10 µM), 1 µL of pure fungal DNA template, 7.4 µL di-deionized water. Real-time PCR was performed in a LineGene K PCR detection system (Bioer, Japan) with the following conditions: 95 °C for 10 min, followed by 35 cycles of 95 °C for 15 s, 67.1 °C for 30 s and 72 °C for 20 s. A standard curve was first constructed using the *A. carbonarius* genomic DNA in the concentration range 10 ng–0.1 pg. This curve was then used as a reference standard for extrapolating quantitative information for DNA targets of unknown concentrations. Real-time PCR amplification reactions were carried out in triplicate from three independent experiments.

Table 1 Fungal strains isolated from different varieties of *Vitis vinifera* (B: Bombino nero; C: Cesanese di Affile; D: Dama; M: malvasia; Mt: merlot; Mo: Montepulciano; Na: Nero d'Avola; Ne: Negroamaro; Rf: Refosco faedis; Rv:Refosco vescovo; S: Sangiovese) harvested in two Italian stations

	Grape varieties																	
Fungi species	B		C		D		M		Mt		Mo		Na		Ne		Rf	Rv	S
Alternaria sp.	x	x			x				x									X	
Aspergillus carbonarius					x				x		x		x		x				
Aspergillus ochraceus									x										
Aspergillus niger																		X	
Cladosporium sp.	x				x		x		x				x		x			X	x
Fusarium sp.									x		x						x	X	
Penicillium sp.															x				
Botrytis cinerea	x				x						x		x				x		x

Statistics

All the experiments (three) were performed in duplicate and the data set (#6 independent data) was used for originating mean values (± SE) whose comparison was performed using the Student's *t* test.

Results

Isolation of fungal strains from grape berries of different varieties of *V. vinifera*

The mycoflora isolated from different grape berries harvested in two Italian stations (Bari and Velletri) are presented in Table 1. *Cladosporium* sp. is the most represented species, being isolated from almost all the grape varieties. *A. carbonarius* was isolated from var. Dama, Negroamaro (Bari) and Nero d'Avola, Merlot, Montepulciano (Velletri).

Conidia growth on the inoculated grape berries

The visual inspection of the grape berries (by a stereo microscope at × 100 magnification) inoculated with one to five conidia each showed that *A. carbonarius* mycelium was not visible up to 24 h after inoculum. The fungus was evident after 40–48 h of incubation. At 120 h, the matrix was completely contaminated by the mycelium that had produced the typical black conidia. The *A. carbonarius* conidia count showed a quick increase between 6 and 48 h, and then the growth rate decreased the next 72 h after berry infection (Figure 1).

Real-time PCR standard curves

The C_t values of the amplification curves of the different solutions of genomic DNA in the concentrations assayed, 10 ng–0.1 pg, ranged from 24 ± 0.3 to 39.8 ± 0.2.

The minimum DNA quantity detectable by the system was 0.2 pg. The linear correlation coefficient R^2 of the standard

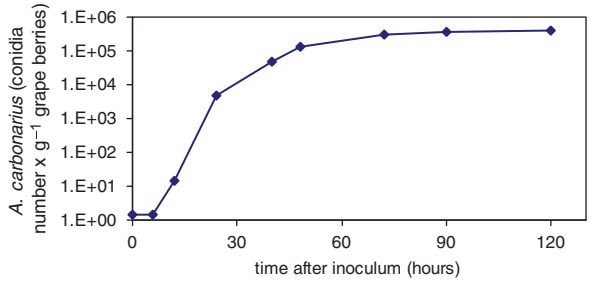

Figure 1 Conidia counting on grapes inoculated with *Aspergillus carbonarius* after 0, 6, 12, 24, 40, 48, 72, 90, 120 h of incubation at 30 °C. The inoculum (1–5 conidia 10 μL^{-1}) was distributed on each grape berry.

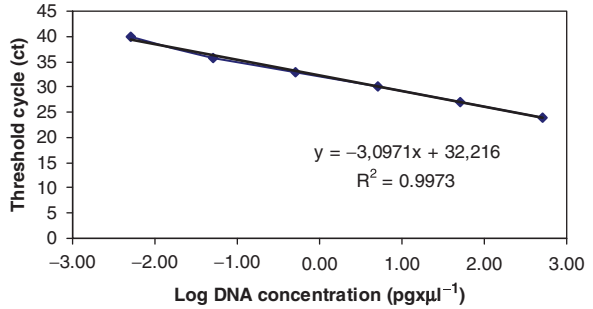

Figure 2 Standard curve is constructed from DNA of known concentration (were used eightfold dilutions of genomic DNA of *Aspergillus carbonarius*, ranging from 10 ng to 1 pg). Each DNA value represents the average of three measurements.

curve was above 0.99 (Figure 2), indicating the accuracy of the method. The slope of the curve was − 3.1, indicating that real-time PCR assays has an amplification efficiency Eff = 1.1.*

SYBR® Green real-time PCR specificity was confirmed by comparing amplification curves using, as a template, the DNA extracted from different fungal strains isolated from grape berries, that is, the naturally contaminating mycoflora. The results (Table 2) showed that positive amplifications were achieved only with DNA from *A. carbonarius*. This suggests that the method developed here allows the early detection of sole *A. carbonarius*.

Detection limits of the qPCR reactions for *A. carbonarius* in artificially inoculated wine grapes

The detection limits of selected primer pairs for detecting *A. carbonarius* strains were analyzed with DNA extracted from artificially inoculated grapes after 0, 6, 12, 24 and 48 h of incubation. The result of *A. carbonarius* detection in this artificially contaminated grape samples (Table 3) showed that the specific primers Acpksfor1/Acpksrev2 allowed a significant ($P < 0.01$) early detection of *A. carbonarius* already after 6 h of incubation.

Early detection of *A. carbonarius* in naturally contaminated grape berries

In Table 4, the DNA quantification of *A. carbonarius* carried out with real-time PCR using the specific primers Acpksfor1/Acpksrev2 in DNA samples of some varieties of grapes harvested in different Italian stations (Bari-Velletri) is shown. The varieties Dama, Merlot, Montepulciano, Negroamaro and Nero d'Avola were significantly positive ($P < 0.01$) for the screening of real-time PCR assay but did not show any evident signs of rotting at the time of harvest (symptomless berries). *A. carbonarius* mycelium developed when the grape berries (from the same bunch) were subsequently incubated for 7 days at 30 °C.

Discussion

In the last decade, it has been shown that OTA contamination from grapes, raisins, wine and wine derivatives was mainly due to *A. carbonarius* (Abarca *et al.*, 2003; Esteban *et al.*, 2004; Battilani *et al.*, 2006). *A. carbonarius* is a dangerous spoilage fungus because the majority (90–100%) of its strains are reported to be potential producers of OTA (Horie, 1995; Terén *et al.*, 1996; Heenan *et al.*, 1998; Cabañes *et al.*, 2002; Serra *et al.*, 2003; Perrone *et al.*, 2006).

Real-time PCR-based methods that target DNA are considered to be a good alternative in comparison with the traditional diagnostic methods used for fungal detection, because of their high specificity and sensitivity.

Mycotoxin biosynthetic-related genes represent useful targets for fungal detection in complex matrices. In relation to this, the *PKS* primer, designed on the OTA-related polyketide synthase gene sequences, has the potential to be adapted to the quantification of mRNA and thus, for monitoring also the expression of OTA biosynthesis-related genes. Their use is well known for the results obtained in correlating fungal development and OTA synthesis in grape vine (Atoui *et al.*, 2007) and in the evaluation of SYBR® Green or TaqMan system suitability in detecting black Aspergilli in grape berries (Selma *et al.*, 2008). This is the first study to examine a similar real-time PCR-based method, i.e. using the PKS primer, to quantify the presence of *A. carbonarius* in Italian grape varieties at different time intervals after inoculum. A similar approach has been discussed

*Amplification efficiency (Eff) was calculated from the slope of the standard curve using the following formula Eff = $10^{-1/\text{slope}}$ -1 (Dorak, 2009).

Table 2 Fungal strain analyzed for A*cpks* primer specificity assays

Strains	Hosts	OTA	Presence of PCR product	Ct SYBR
Aspergillus carbonarius 993[1]	*V. vinifera*	–	+	25.02 ± 0.28
A. carbonarius 2034[1]	*V. vinifera*	±	+	24.96 ± 0.31
A. carbonarius M[2] (item not yet assigned)	*V. vinifera*	+	+	25.00 ± 0.16
A. carbonarius C#1	*V. vinifera*	+	+	25.13 ± 0.23
A. carbonarius C#2	*V. vinifera*	+	+	24.67 ± 0.04
A. carbonarius C#3	*V. vinifera*	+	+	24.78 ± 0.19
A. carbonarius C#4	*V. vinifera*	+	+	24.46 ± 0.16
A. carbonarius C#5	*V. vinifera*	+	+	24.65 ± 0.27
Fungal mixtures[3]	*V. vinifera*	+	+	33.00 ± 0.45
Fungal mixtures[3] *without A. carbonarius*	*V. vinifera*	–	–	≥41
Aspergillus niger ITEM 7096[2]	*V. vinifera*	+	–	≥41
A. niger IBT 19349[4]	Unknown	+	–	≥41
A. niger ITEM 4717[2]	*V. vinifera*	–	–	≥41
A. niger 1034[1]	*V. vinifera*	–	–	≥41
Aspergillus tubingensis ITEM 4709[2]	*V. vinifera*	±	–	≥41
Aspergillus ochraceus#	*V. vinifera*	–	–	≥41
Cladosporium sp#	*V. vinifera*	–	–	≥41
Aspergillus flavus NRRL 3357[5]	*V. vinifera*	–	–	≥41
Aspergillus parasiticus NRRL 2999[5]	*V. vinifera*	–	–	≥41
Botrytis cinerea[6]	*V. vinifera*	–	–	≥41
Penicillium sp[6]	*V. vinifera*	–	–	≥41
Fusarium sp[6]	*V. vinifera*	–	–	≥41
Penicillium expansum[5]	*V. vinifera*	–	–	≥41
Non-template control			–	41.35 ± 0.30

PCR and real-time PCR amplification results with the specific primers Acpksfor1/Acpksrev2. The assay was carried out using DNA extracted from various grape berries-contaminating fungi.

[1]Strains supplied by Prof. P. Battilani (Univerity of Piacenza 'Cattolica', Italy).

[2]Fungi collection present in the ISPA-CNR, and collected in the area of Manduria, Bari, Italy.

[3]*A. niger*, *A. ochraceus*, *B. cinerea*, *A. Carbonarius*.

[4]VTT Culture Collection.

[5]Strains supplied by Prof. C. Fanelli (University of Rome 'Sapienza', Italy).

[6]Strains isolated in this work.

±, samples showed a low production of OTA and/or a low efficiency in PCR amplification.

Table 3 DNA quantification of *Aspergillus carbonarius* inoculated on grape berries after 6, 12, 24, 48 hours of incubation at 30 °C

Time of incubation after inoculum (h)	*A. carbonarius*	
	Ct	DNA (pg)
6	38.11 ± 0.15	2.5E-01
12	35.19 ± 0.18	2E+00
24	28.00 ± 0.27	5E+02
48	24.49 ± 0.20	6E+03
Non-inoculate grape	41.65 ± 0.46	
Non-template control	41.35 ± 0.30	

The quantification was carried out by comparison with standard curves. The data are the mean ± SD of three separate experiments.

recently (González-Salgado *et al.*, 2009), but in this case, 16 h is the minimum time necessary to obtain a signal using a different subset of primers (based on the multicopy ITS) and an higher inoculum amount (10^3 conidia g^{-1} grape berries) than in

Table 4 DNA quantification by SYBR® Green real-time PCR was carried out by comparison with standard curves on naturally contaminated grapes

Grape cultivars	*Aspergillus carbonarius* DNA (pg/mg grape berry)
Bombino nero	–
Cesanese	–
Dama	0.79 ± 0.02
Malvasia	–
Merlot	0.39 ± 0.05
Montepulciano	0.30 ± 0.07
Negroamaro 1	1.41 ± 0.09
Negroamaro 2	0.47 ± 0.03
Nero d'Avola	0.29 ± 0.01
Refosco vescovo	–
Refosco faedis	–
Sangiovese	–

The data are the mean ± SD of three separate experiments.

the present study. Further, the DNA extraction procedure developed here avoids the use of a stomacher (Selma *et al.*, 2008) or of an expensive miniprep kit (Atoui *et al.*, 2007).

In this study, it has been shown that the specific primers Ac*pks*, newly designed on the OTA biosynthesis-related Ac*KS10* gene sequence, are able, under the experimental conditions described herein, to detect the presence of *A. carbonarius* after only 6 h of a minimal inoculum (1–5 conidia g^{-1} grape berries), i.e. when the visual inspection (stereo microscope) did not yet allow detection.

This study is, therefore, functional for the early detection *A. carbonarius* in grape berries for a quantity of genomic DNA estimated at 0.33 pg g^{-1} of berries. The results showed that around 1–5 conidia g^{-1} grape berries are enough to yield a positive result with SYBR® Green real-time PCR amplification 6 h after inoculum. This result can be considered an improved detection methodology in comparison with similar studies wherein a minimum threshold of 10^3 conidia is required for the accurate quantification of *A. carbonarius* in grapes (Selma *et al.*, 2008; González-Salgado *et al.*, 2009).

In addition, in this work, the real-time PCR assay allows the quantification of *A. carbonarius* in naturally contaminated grapes and thus can be utilized directly as an *in vivo* assay, i.e. from grape berries taken directly from the field (no need for further incubation step). The results show the ability of this system to early detect the natural presence of the fungus in grape because in a working day, it is possible to harvest the berries, extract high-quality DNA and perform real-time PCR amplifications. In fact, the grape berries in which *A. carbonarius* DNA was present at a detectable level, although not as yet symptomatic, showed, after proper incubation, the presence of the fungus, confirming the result obtained by the molecular tool developed here.

Regarding food safety concerns, recent studies (Atoui *et al.*, 2007) have correlated the quantity of *A. carbonarius* DNA with OTA levels in grape berries stating that 10 ng DNA g^{-1} grape berries are sufficient to achieve the maximum amount allowed under the European Union regulation. In this work, the maximum level of the *A. carbonarius* DNA found in the assayed varieties was 1.41 ng DNA g^{-1} grape berries (var. Negroamaro), showing that the system also allows the detection, *in vivo*, of the amount of fungus potentially sufficient to produce health dangerous OTA levels.

The molecular detection technique developed in this work represents a contribution to the early detection of fungal contamination in important food matrices such as grape berries.

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