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ORIGINAL ARTICLE

Polymerase chain reaction detection of *Aspergillus carbonarius* in grapes at different time after inoculum

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Abstract

Objectives The aim of this research was to set up a polymerase chain reaction (PCR)-based method to early investigate the presence of *Aspergillus carbonarius* in grapes. Polymerase chain reaction-based methods that target DNA are considered a good alternative with respect to traditional diagnostic methods for the early detection of pathogens because of their high specificity and sensitivity. *Methods* In this study, two polymerase chain reaction assays to detect the presence of *A. carbonarius* in grapes have been developed by using species-specific primers designed on the basis of Internal Transcribed Spacers of rDNA units and on a polyketide synthase gene responsible for ochratoxin A biosynthesis. Primers specificity was evaluated by polymerase chain reaction on *A. carbonarius* DNA as template and then on DNA extracted from *A. carbonarius* – contaminated and uncontaminated grape berries. The developed polymerase chain reaction method was used to analyze the detection limits of *A. carbonarius* DNA directly on grapes. *Results* Polymerase chain reaction amplification allows a clear detection of *A. carbonarius* DNA 24 hours after inoculum on grapes, i.e. when the fungus is not yet visible. *Conclusion* Early detection by polymerase chain reaction of the ochratoxin A producer fungus *A. carbonarius* could help to achieve an effective control of the fungal contamination, particularly concerning mycotoxigenic fungi.

Introduction

Aspergillus carbonarius is an important ochratoxin A (OTA) producing fungus, which is the main fungus responsible for OTA contamination of grapes and wine. OTA is a secondary metabolite, produced by fungi belonging to *Aspergillus* and *Penicillium* genera with nephrotoxic, nephrocarcinogenic, teratogenic and immunosuppressive effects in animals and probably in humans (Varga & Kozakiewicz, 2006). The International Agency for Research on Cancer has classified OTA as a group B carcinogen, that is, a possible carcinogen to humans. As a consequence, the European Commission has imposed regulatory limits for the maximum tolerable presence of this toxin in different foodstuffs (Commission Regulation 1881/2006). After cereals, wine is considered a major source of daily OTA intake (Cabañes *et al.*, 2002). Many authors have underlined the contribution of *A. carbonarius* to the ochratoxin contamination of wine and grape (Accensi *et al.*, 2001; Pietri *et al.*, 2001; Battilani & Pietri, 2002; Serra *et al.*, 2003). From these works it emerges that this species is very invasive, colonizes berries and that temperature, rain and relative humidity are the main factors that influence OTA production in grapes.

The interest in this toxin is confirmed by the high number of reports published during the last 10 years and by the development of different analytical methods to obtain precise and accurate quantification from different food matrices (Zimmerli & Dick, 1996; Festas *et al.*, 2000; Visconti *et al.*, 2000; Markaki *et al.*, 2001; Soleas *et al.*, 2001).

In recent years, it has been revealed that OTA contamination in grape, raisin, wine and wine derivatives is due mainly to *Aspergillus niger* and *A. carbonarius* (aka black Aspergilli)

(Abarca *et al.*, 2003; Esteban *et al.*, 2004). *A. carbonarius* is not as widespread as *A. niger* but it is equally dangerous because most of its strains are toxigenic and have the highest ochratoxigenic potential. These pathogens can also cause decay and deterioration in stored products (Horie, 1995; Téren *et al.*, 1996; Heenan *et al.*, 1998; Cabañes *et al.*, 2002).

To prevent OTA contamination in foodstuffs, such as cereals, coffee or grape, it is necessary to have a rapid and specific method to early detect OTA producing fungi (Dao *et al.*, 2005) in order to predict the risk of mycotoxin contamination in food (Atoui *et al.*, 2007).

The use of polymerase chain reaction (PCR) in foodstuffs is increasing because of its ability to identify fungal species unambiguously, without the isolation and identification steps proper of the classical microbiological approach; furthermore this molecular technique allows quantifying fungal contamination through the evaluation of DNA content.

Further studies may lead to the use of PCR-based systems to decide whether a lot of foodstuffs may be put on the market or not.

It is known that the contamination of agricultural commodities with mycotoxins can occur without visible fungal contamination; therefore, in this work we have developed a PCR-based method for detecting very low quantity of *A. carbonarius* DNA in artificially inoculated grape berries; furthermore a semi-quantitative detection of *A. carbonarius* by designing new primers in combination with a rapid and efficient fungal DNA extraction procedure has been approached. These methods will contribute to lower the risk of OTA contamination by *A. carbonarius* in wine.

Materials and methods

Grape berries contamination

A. carbonarius M (item not yet classified on fungi collection ISPA-CNR, Institute of Sciences of Food Production) or *Aspergillus niger* ITEM 7096 (fungi collection ISPA-CNR) conidia were collected from culture tube with 0.01% (v/v) Triton (Sigma Aldrich, Saint Quentin Fallavier, France) and a conidia suspension (10^5 conidia mL^{-1}) in sterile distilled H_2O was realized. In a sterile room, from three to five berries, superficially sterilized (by clorex 2% washings), were placed on a 6 cm Petri plate; 2×10^3 conidia 20 μL^{-1} of *A. carbonarius* or *Aspergillus niger* were distributed to each berry. The plates were incubated at 30 °C for 24, 48, and 120 h.

Grape berries DNA extraction

Fresh grape berries (300 mg), contaminated with *A. carbonarius* or uncontaminated, were homogenized with mortar and pestle in liquid nitrogen and incubated with 1 mL extraction buffer [200 mM Tris-HCL (Sigma Aldrich) pH 8.5, 250 mM NaCl (Sigma Aldrich), 25 mM EDTA (Sigma Aldrich), 0.5% SDS] for 60 min at 65 °C under constant agitation in a orbital shaker. After incubation, samples were put on ice for 10 min and centrifuged at 12 000 rpm for 15 min at 4 °C. The supernatant was collected in a 2 mL Eppendorf tube and 3/10 volume of 4 M sodium acetate was added. This solution was placed on ice for 30 min and centrifuged at 12 000 rpm for 10 min at 4 °C and the supernatant was transferred to a new Eppendorf tube. One volume of phenol–chloroform–isoamyl alcohol (25:24:1) was added and sample mixed for 5 min and centrifuged at 12 000 rpm for 10 min at 4 °C. The upper aqueous phase was transferred into another tube, adding 1 volume of chloroform and sample mixed for 5 min and centrifuged at 12 000 rpm for 15 min at 4 °C. The upper aqueous phase was transferred into another tube, adding 0.5 volume of cold 2-propanol, two to three times to invert the tube leaving the DNA precipitated for 20 min at −20 °C. Samples were centrifuged at 14 000 rpm for 15 min at 4 °C. The pellet was washed with 0.5 mL of ethanol and after centrifugation at 14 000 rpm for 15 min at 4 °C was dissolved in 28 µL of sterile H_2O and 2 µL of RNAsi 20 mg mL^{-1} for 30 min at 37 °C. The same procedure (except for the sodium acetate phase) was performed for extracting DNA from 30 mg of lyophilized mycelium of *A. carbonarius*.

Primers design and amplification conditions

A. carbonarius specific primers Acpksfor/Acpksrev2, reported in Table 1, were derived from a conserved region in the β-ketosynthase domain of a *pks* gene from *A. carbonarius* (GenBank accession number: AY540952). The primer pair was designed using the Primer Express software v2.0 and located at positions 104–124 and 414–434, respectively.

Table 1 *Aspergillus carbonarius*-specific primers

Primer	Forward 5′–3′	Reverse 5′–3′	Size of Polymerase chain reaction product (bp)
Acpksfor/Acpksrev2	GCAGCGGGAGTCAATGTAAT	GCGTCGTACAAAGCCTCTT	330
AclTSfor2/AclTSrev1	GTGAAGTCTGAGTCGATTGTT	GGAAAAAAAGGTTGGAGTT	239

The primers amplify a 330 bp fragment that is specific for *A. carbonarius*.

The primers AcITS are designed on the basis of Internal Transcribed Spacers of rDNA units (ITS) of *A. carbonarius*. These primers were obtained by comparing the sequences ITS1-5.8S-ITS2 of different species of *Aspergillus* including *A. carbonarius, A. niger, A. ochraceus* from different Italian geographic areas. PCR amplification conditions using these primers were: 1 cycle of 2 min at 94 °C, 30 cycles of 45 s at 94 °C (denaturation), 1 min at 67.1 °C (primer Acpksfor/Acpksrev2) or 66 °C (primer AcITS), 1 min at 72 °C (extension) and finally 1 cycle of 7 min at 72 °C. The amplification reaction was carried out in final volumes of 25 µL containing 100 ng of DNA and PCR products were detected in 1% agarose ethidium bromide gels in TAE 1 × buffer.

Results

Primer specificity and sensitivity

DNA extracted from *A. carbonarius* and *A. niger* was positively amplified with Acpksfor/Acpksrev2 primers (Figure 1A), whereas only the former gave a positive result with AcITS primers (Figure 2A).

A test of the method sensitivity with serial dilutions (range 1 pg–100 ng) of fungal DNA with both selected primers was carried out. The relative luminescence intensity

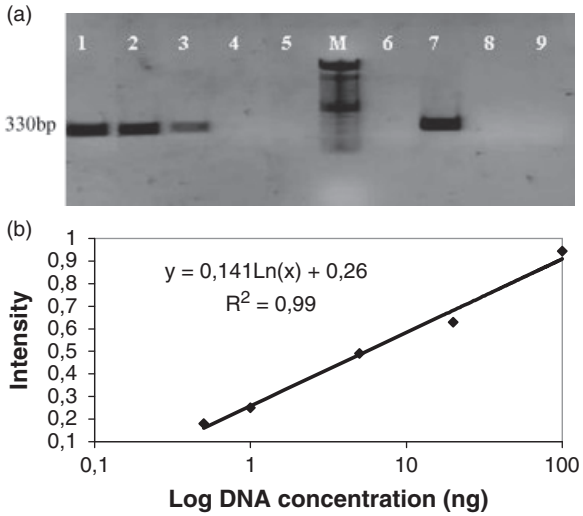

Figure 1 (A) 1% of agarose gel electrophoresis of polymerase chain reaction (PCR) fungal DNA with primers Acpksfor/Acpksrev2: (1) *Aspergillus carbonarius* (manduria); (2) *A. carbonarius* 993; (3) *Aspergillus niger* 7096; (4) *Aspergillus ochraceus*; (5) *Cladosporium* sp.; (M) 100 bp DNA ladder (Invitrogen, San Giuliano Milanese, Italy); (6) *Penicillium* sp.; (7) *A. niger* 4717; (8) *Alternaria* sp.; (9) PCR control. (B) PCR Amplification obtained from genomic DNA serial dilutions of *A. carbonarius* with primers Acpksfor/Acpksrev2.

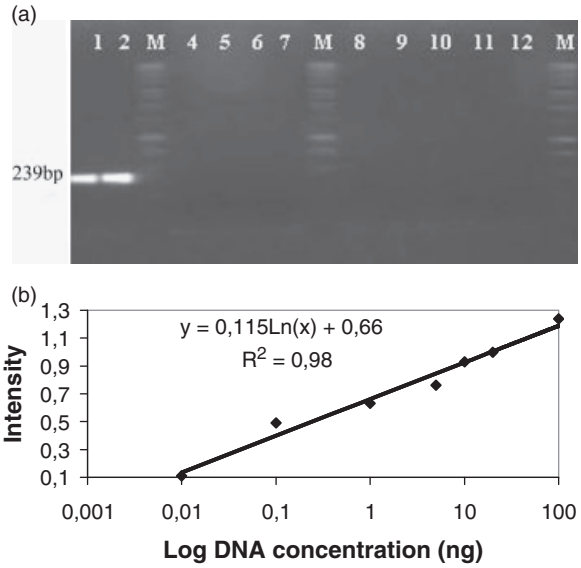

Figure 2 (A) 1% of agarose gel electrophoresis of polymerase chain reaction (PCR) fungal DNA with primers AcITSfor2/AcITSrev1; (1) *Aspergillus carbonarius* (manduria); (2) *A. carbonarius* 993; (4) *Aspergillus niger* 7096; (5) *A. niger* 4717; (6) *A. niger* 4709; (7) *Alternaria* sp.; (8) *Cladosporium* sp.; (9) *Aspergillus ochraceus*; (10) *Fusarium* sp.; (11) *Penicillium* sp.; (12) PCR control; (M) 1 kb DNA ladder (Fermentas, St. Leon-Rot, Germany); (B) PCR Amplification obtained from genomic DNA serial dilutions of *A. carbonarius* with primers AcITSfor2/AcITSrev1.

of the different quantity of fungal genomic DNA was quantified by using the software UVI-Doc Mw Version 10.01 and these data were used to generate a relative luminescence intensity standard curve (semi-quantitative analysis). The amplification of *A. carbonarius* DNA with both primers in a 1 pg–100 ng range was carried out. The results show that the sensitivity was 10 pg μL^{-1} when AcITS primers were used and it was 500 pg μL^{-1} with Acpksfor/Acpksrev2 primers. The regression curves, generated with the different relative luminescence intensity values (Figures 1B and 2B) show a positive and good correlation ($R^2 = 0.97$; 0.98) between intensity and DNA amount and the function [1B: Intensity = 0.141 × ln(DNA) + 0.26], [2B: Intensity = 0.115 × ln(DNA) + 0.66] of this correlation, give us the chance to attempt a semi-quantification of other DNA samples.

PCR detection of grape berries contaminating-fungi

The stereo microscope inspection (× 100) of the contaminated grape berries, showed that the presence of the two toxigenic fungi is not evident at 24 h after inoculum; at 48 h the fungal development is evident (abundant white

mycelium) and at 120 h the matrix is completely contaminated by the mycelium and typical black conidia (Figure 3A).

The results of the PCR amplification (Figure 3B) of 100 ng of total DNA extracted from grape berries, uninoculated and inoculated with *A. carbonarius* after 24, 48 and 120 h of incubation show a clear amplification signal already after 24 h of incubation time, i.e. when it is not yet possible the fungal detection using a stereo microscope ($\times 100$). PCR results with primers AcITS confirm the results with primers Acpksfor/Acpksrev2 (data not shown). The absence of amplification with primers chosen from the DNA extracted from grape berry undergo sterilization, it is confirmation that the matrix does not affect the PCR analysis.

The results of the semi-quantitative analysis (obtained by matching relative luminescence intensity of the samples with the DNA standard curve) showed a quantity *A. carbonarius* DNA at 24 h of 1.3×10^6 pg, at 48 and at 120 h of 9.0×10^6 and 3.6×10^7 pg, respectively.

The results of PCR using DNA extracted from samples inoculated with *A. niger* (Figure 4A), show an amplification, i.e. when the fungus was evident at 120 h after inoculum (Figure 4B). These results indicate that the Acpksfor/Acpksrev2 primers are specific for an early detection of *A. carbonarius* from grapes whereas *Aspergillus niger* generates amplicons evident only at a very advanced stage of fungal infection (120 h).

Discussion

The PCR results using DNA extracted from various grape-contaminating fungi, show that only *A. carbonarius* DNA was amplified with AcITS, whereas *A. carbonarius* and *A. niger*, both black Aspergilli, were amplified with Acpksfor/Acpksrev2 primers. None of the other assayed species, belonging to other *Aspergillus* spp. and to *Penicillium*, *Cladosporium*, *Botrytis* and *Alternaria* spp., gave a positive result with these primers. PCR-based methods, which target DNA are considered a good alternative in comparison with traditional diagnostic methods for early detection because of their high specificity and sensitivity.

Recently, several studies with PCR assays have been focused on the detection and quantification of *A. carbonarius*, which is the fungal pathogen mainly responsible for producing and releasing the OTA on grapes and wine (Battilani & Pietri, 2002; Cabañes *et al*., 2002). One of the most important factors in the development of such methods is the reliability of the primer set and the targeted DNA sequence for the organism of interest (Dao *et al*., 2005). In the last few years the detection and quantification of *A. carbonarius* has been achieved using PCR-based assays with primers based on the acyltransferase domain of polyketide synthase (Atoui *et al*., 2006; Mulè *et al*., 2006; Selma *et al*., 2008) and ITS sequence (Patiňo *et al*., 2005). ITS

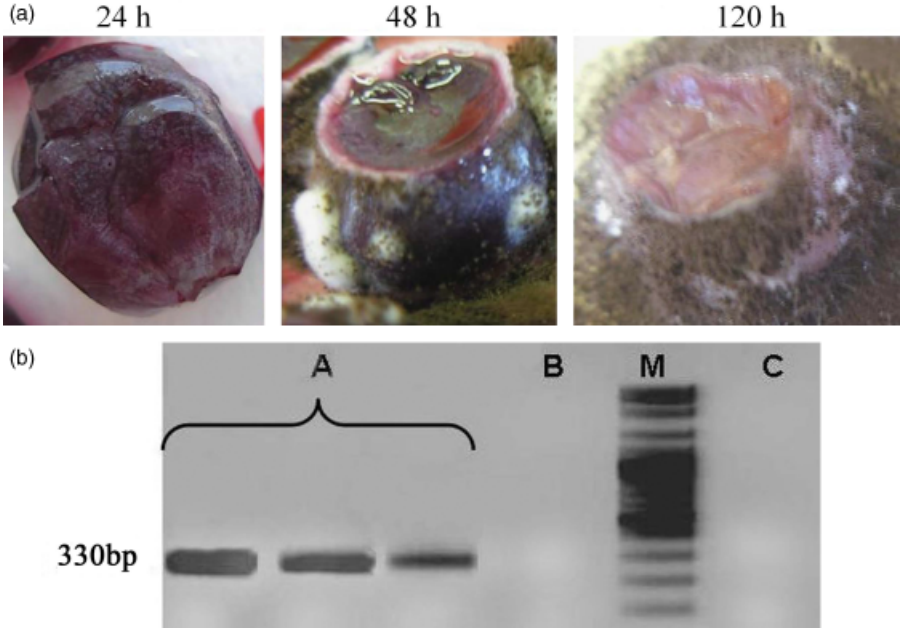

Figure 3 (A) Inoculated wine grapes with *Aspergillus carbonarius* after: 24, 48, 120 h. (B) 1% of agarose gel electrophoresis of DNA wine grapes polymerase chain reaction (PCR) with primers Acpksfor/Acpksrev2: (A) inoculated grapes with *A. carbonarius* (manduria) after, 120, 48, 24 h, (B) uninoculated grapes, (M) 100 bp plus DNA ladder (Fermentas); (C) PCR control.

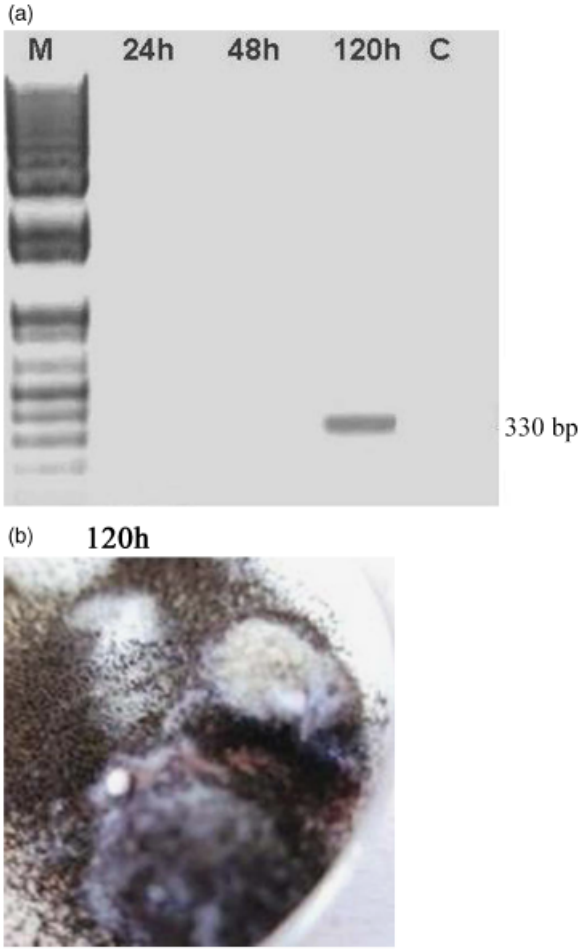

Figure 4 (A) 1% of agarose gel electrophoresis of polymerase chain reaction (PCR) fungal DNA with primers Acpksfor/Acpksrev2 inoculated grapes with *Aspergillus niger* after 24, 48, 120 h: (C) PCR control; (M) 1 kb DNA ladder (Invitrogen). (B) Inoculated wine grapes with *A. niger* after 120 h.

regions of rDNA units are high variability regions and particularly useful when it is necessary to discriminate between species and to carry out phylogenetic and population studies in filamentous fungi (Henry *et al*., 2000; Zhao *et al*., 2001). To our knowledge, this is the first study to examine this PCR-based method for the detection and semi-quantification of the development of *A. carbonarius* in grapes at different time intervals after inoculum, i.e. during the fungal growth on the contaminated food stuff.

In this study the minimum amount of DNA detected by this PCR-based method was 500 pg obtained by using pks primers and 10 pg with AcITS primers. These results confirm the detection limits of ITS amplification identified in other papers (Bluhm *et al*., 2002; Patiňo *et al*., 2005) and the detection limit we obtained with the primer Acpksfor/Acpksrev2 with plasmid DNA is an excellent result compared with that obtained by other authors using the SYBR Green in Real Time PCR on genomic DNA (Atoui *et al*., 2007). Linear regression analysis of intensity corresponding to different concentrations of fungal DNA yielded R^2 values above 0.97, demonstrating the accuracy of the method. This PCR analysis was successfully used to detect *A. carbonarius* in grape berries at 24 h after inoculum with 2×10^3 conidia 20 μL^{-1} of *A. carbonarius*.

These results show that molecular techniques developed in this work, represent a concrete contribution to the prevention of fungal contamination, allowing fungal detection from grape when other inspection methods do not work.

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