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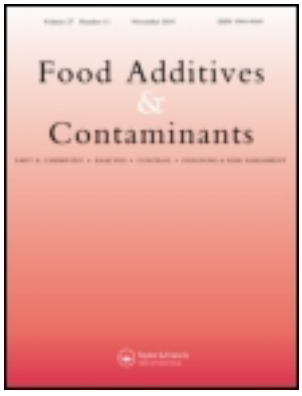

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Early detection of *Aspergillus carbonarius* and *A. niger* on table grapes: a tool for quality improvement

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Early detection of *Aspergillus carbonarius* and *A. niger* on table grapes: a tool for quality improvement

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Aspergillus carbonarius and *A. niger* aggregate are the main fungal contaminants of table grapes. Besides their ability to cause black rot, they can produce ochratoxin A (OTA), a mycotoxin that has attracted increasing attention worldwide. The objective of this work was to set up a simple and rapid molecular method for the early detection of both fungi in table grapes before fungal development becomes evident. Polymerase chain reaction (PCR)-based assays were developed by designing species-specific primers based on the polyketide synthases (PKS_S) sequences of *A. carbonarius* and *A. niger* that have recently been demonstrated to be involved in OTA biosynthesis. Three table grape varieties (Red globe, Crimson seedless, and Italia) were inoculated with *A. carbonarius* and *A. niger* aggregate strains producing OTA. The extracted DNA from control (non-inoculated) and inoculated grapes was amplified by PCR using ACPKS2F-ACPKS2R for *A. carbonarius* and ANPKS5-ANPKS6 for *A. niger* aggregate. Both primers allowed a clear detection, even in symptomless samples. PCR-based methods are considered to be a good alternative to traditional diagnostic means for the early detection of fungi in complex matrix for their high specificity and sensitivity. The results obtained could be useful for the definition of a 'quality label' for tested grapes to improve the safety measures taken to guarantee the production of fresh table grapes.

Keywords: molecular biology; polymerase chain reaction (PCR); high-performance liquid chromatography (HPLC); mycotoxins; fungi; ochratoxin A; fruit

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Introduction

Many fungi can occur on table grapes in the vineyard, but the main concern from the point of view of mycotoxin contamination is represented by the group of black *Aspergillus* species, in particular *A. carbonarius* and *A. niger* aggregate (Ailsa et al. 2007). They are Deuteromycota fungi (imperfect fungi, asexual reproduction) that belong to the Order Hyphomycetes, Family Moniliaceae, section Nigri (Atoui 2006). In addition to their ability to cause black rot diseases in the field, they can produce ochratoxin A (OTA), a mycotoxin that is attracting increasing attention worldwide (Varga and Kozakiewicz 2006).

OTA, the most toxic of the ochratoxins, is produced by several fungal species belonging to *Aspergillus* and *Penicillium* genera (Varga and Kozakiewicz 2006). It has a significant economic impact and poses a serious problem for human health. It is named after *A. ochraceus*, the first rot agent from which it was isolated (Van der Merwe et al. 1965), although later on other genera were reported to be capable of producing this toxin. The compound 7-carboxyl-5-chloro-3,4-dihydro-8-hydroxyl-3*R*-methylisocoumarin-7-L-phenylalanine ($C_{20}H_{18}ClNO_6$), usually known as OTA, can be found in several food and drink commodities, including cereals, coffee and wine, at different levels (Battaglia et al. 1996).

Several studies have been performed to investigate contamination with black aspergilli on wine grapes (Battilani et al. 2004; Leong et al. 2006), but only a few works have been carried out on table grapes (Guzev et al. 2006). In this work *A. carbonarius* and *A. niger* aggregate strains able to produce OTA were frequently isolated from table grapes.

Most research efforts were concentrated on the means of prevention of OTA biosynthesis as the best defence for protecting consumers. However, prevention is not always possible, especially from OTA biosynthesis occurring under field conditions. In fact, OTA remains in processed food, and the recovery of

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infected products is only possible through their decontamination (Bellì 2006). Conversely, in the case of table grapes, prevention remains the only possible way to control OTA formation. This point can be achieved only thanks to the early detection of the OTA-producer fungus.

Traditional diagnostic methods used in food mycology are based on macroscopic and microscopic features and culture in appropriate media. On the one hand, the development of fruiting structures requires 2–10 days of culture on different media (Raper and Fennell 1965), increasing considerably the time of analysis. On the other hand, these methods have shown a low degree of sensitivity and are difficult to standardize (Zhao et al. 2001). Misidentification can often occur because some fungi may be poorly characterized or because considerable expertise is required (Patiño et al. 2005).

The increasing use of molecular methods in fungal diagnosis has provided tools for answering taxonomic questions that morphological procedures have left unsolved (Taylor et al. 1999; Parenicova et al. 2000). Polymerase chain reaction (PCR)-based methods that target on DNA are considered a good alternative for rapid and early diagnosis because of their high specificity and sensitivity (Bluhm et al. 2002).

Reduction of OTA contamination may be accomplished by the early detection of OTA-producing fungal species in order to intervene at the right time to reduce the source of OTA.

Several approaches have been followed for the molecular detection of the OTA-producing fungi. Atoui et al. (2006) developed a specific PCR assay for the detection of *A. carbonarius* in grapes, grape juices and wines.

González-Salgado et al. (2009) set up a specific detection method of some *A. carbonarius* strains isolated from Portugal by SYBR Green and TaqMan quantitative PCR assays based on the multicopy ITS2 region of the rRNA gene to be used in wine grapes. The primers and the TaqMan probe were based on the ITS 2 multicopy region. The specificity and sensitivity of both assays were tested on genomic DNA mixtures of several *A. carbonarius* strains and other fungal species frequently present in grapes. Both methods were also compared using grapes inoculated with different spore concentrations of *A. carbonarius*, detecting up to 0.4 pg DNA g^{-1} of berries.

As for *A. niger*, no data have been found so far in the literature for the detection and quantification using real-time PCR assays on grapes.

For this reason, the objective of this work was to set-up a simple, rapid, specific, and sensitive molecular method for the early detection of *A. carbonarius* and *A. niger* aggregate in table grapes before fungal development becomes evident.

Materials and methods

Fungal strains and culture conditions

Each grape sample (approximately 500 g) was washed with 500 ml of sterile distilled water on a shaker for 30 min. Aliquots (100 µl) of washing water and its serial dilution were spread on Czapek–Dox–agar (CDA) medium with 0.02% streptomycin sulphate. The plates were incubated at 25°C and checked daily until the formation of fungal colonies. Each colony was transferred to a Petri dish with the same medium and incubated for 7 days at 25°C. *Aspergillus* strains were identified according to their morphological characteristics (macroscopically and microscopically), following the rules and descriptions provided by Raper and Fennell (1965) and Pitt and Hocking (1985), as well as following molecular tools. Strains were also tested to determine their ability to produce OTA. The black aspergilli strains were preserved in slants with CDA medium at 4°C.

All strains used in this study were OTA producers. The different strains were isolated from grapes in the Apulia region of Italy. Fungal cultures were maintained on CDA medium and stored on the same medium at 4°C.

Infection of grapes

Berries from three table grape varieties, Red globe, Crimson seedless, and Italia, were collected during harvesting time and stored at 4°C. Grapes were superficially sterilized with sodium hypochlorite at 2% v/v for 2 min, rinsed with sterile distilled H_2O, and incubated in a container at 30°C after superficial inoculation by 20 µl of conidia suspension (2.5 CFU μl^{-1}) prepared from an OTA-producer strain of *A. carbonarius* or *A. niger* aggregate. Four different experiments were performed during 40 days of post-harvest storage: at harvest and after 13, 26 and 40 days of conservation. Inoculated and un-inoculated berries samples were collected 0, 3, 6, 9 and 12 days after inoculation and stored at −80°C until the day of analysis.

DNA extraction from fungal strains

A. carbonarius and *A. niger* aggregate strains were cultured in 100 ml Erlenmeyer flasks containing 20 ml potato dextrose broth with four to five mycelial disks cut from the border of 4–5-day-old colonies on PDA plates, and incubated at 26°C on a shaker at 120 rpm. Mycelia from 4-day-old cultures were harvested by filtration through Whatman No. 1 paper and kept at −20°C for DNA extraction. A total of 5–10 mg of the conserved mycelia were collected with a spatula, suspended in 400 µl of breaking buffer and extracted with 400 µl of phenol/chlorophorm/isoamyl alcohol (25 : 24 : 1 v/v/v) in the presence of 25 mg of acid-washed

Table 1. Sequence of degenerated primers for *Aspergillus* spp. (PKS1) and specific primers for *A. carbonarius* (ACPKS2).

Primer	Forward 5′–3′	Reverse 3′–5′	Fragment (bp)
PKS1	GATGAGAA(CT)GC(TA)GATGG(GC)T	TCGCCGA(GA)(TC)TGGGTGCCGGTGCC	267
ACPKS2	GCAGCGGGAGTCAATGTAAT	GCGTCGTACAAAGCCTCTT	330

glass beads (425–600 μm diameter) and two sterile 5 mm stainless steel balls. This mixture was vortexed at 3000 rpm for 10 min, and centrifuged for 15 min at 13,000 rpm. The supernatant was mixed with an equal volume of chloroform/isoamyl alcohol (24 : 1 v/v), vortexed at 3000 rpm for 2 min, centrifuged for 5 min at 13thinsp;000 rpm, and precipitated with 2 vols of 100% cold isopropanol (−20°C). The precipitated DNA was washed with 70% (−20°C) ethanol, dissolved in 50 μl of TE buffer (10 mM Tris-HCl, 0.1 mM ethylenediamine tetra-acetic acid (EDTA), pH 7.6) and quantified using a spectrophotometer (Yaseen et al. 2004).

DNA extraction from grape samples

An aliquot of 250 mg of lyophilized homogenized grapes was suspended in 1 ml of lysis buffer (200 mM of Tris-HCl pH 8, 25 mM of NaCl, 25 mM of EDTA and 0.5% of sodium dodecylsulphate (SDS)) and vortexed at 3000 rpm for 10 min. After 1 h at 65°C, and 10 min in ice, the sample was centrifuged at 13,000 rpm at 4°C for 15 min. The aqueous phase was mixed with 200 μl of sodium acetate 4 M, put in ice for 30 min and centrifuged at 13,000 rpm at 4°C for 15 min. The recovered supernatant was homogenized with an equal volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1 v/v/v), vortexed for 5 min and centrifuged at 13,000 rpm at 4°C for 10 min. An equal volume of chloroform 100% was added to the aqueous phase, then gently vortexed for 5 min and centrifuged at 12,000 rpm at 4°C for 15 min.

The DNA contained in the aqueous phase was precipitated by adding 0.5 vol. of isopropanol and stored for 20 min at −20°C. The pellet obtained after 15 min of centrifuge at 13,000 rpm at 4°C was washed with ethanol 70% and centrifuged at 13,000 rpm for 15 min at 4°C. The pellet obtained was dried, dissolved in 28 μl of sterile distilled H_2O and 2 μl of RNAse (20 mg ml^{-1}) and stored at 37°C for 30 min.

Primer design and PCR reaction

A. carbonarius-*specific primer*

PKS genes encode the polyketide synthase (PKS) enzyme, which is involved in the biosynthesis of secondary metabolites. The gene bank has only five partial sequences of the *A. carbonarius* PKS gene (Atoui et al. 2006). On the basis of these sequences, a degenerated primer (PKS1) for *Aspergillus* spp. and a specific primer for *Aspergillus carbonarius* (ACPKS2) were designed (Table 1).

A. niger-*specific primer*

The degenerated primer for *Aspergillus* spp. PKS1 was used to amplify a partial sequence of the *PKS* gene of *A. niger* (Yaseen et al. 2009). On the basis of this sequence, a couple of primers have been designed to amplify a DNA fragment of 157 bp: ANPKS 5 (5′-ACGGTAAACGTCCTGGATGA-3′) and ANPKS 6 (3′-CGTGCTGTTGAAGCCACTT-5′).

PCR amplification

Specific PCR assays were developed in this study for the detection of both *A. carbonarius* and *A. niger*, the main source of OTA contamination on table grapes. To demonstrate the specificity of these primers, PCR amplification of genomic DNA from various fungal strains was performed; PCR was executed in a 25 μl mixture containing 100 ng of genomic DNA, 5 μl of 5× Colorless Go Taq Flexi buffer (Promega, Madison WI, USA), 100 μM of dNTPs buffer (Promega), 2.5 mM $MgCl_2$ (Promega), 10 μM each of forward and reverse primers, and 2.5 unit of Go*Taq*® DNA polymerase (5 U μl^{-1}; Promega). The reactions were performed under the following PCR conditions: 95°C for 300 s; 35 cycles at 95°C for 10 s, 62°C for 10 s for *A. carbonarius* and 52°C for *A. niger*, 72°C for 15 s; plus a final extension cycle at 72°C for 300 s. To visualize the amplification of the expected size fragment, 8 μl of each PCR sample were analysed by agarose gel electrophoresis. Amplicons were separated by electrophoresis in 1.5% agarose gels in TAE buffer (1×) at 80 V for 10 min followed by 100 V for 45 min, and stained for 10 min with ethidium bromide (2 μg ml^{-1}).

Quantitative detection by real-time PCR

A real-time PCR method was developed using SYBR Green dye to detect *A. carbonarius* and *A. niger* OTA-producing strains on table grapes. DNA extractions were performed from 191 samples (from the three tested varieties), and subject to real-time PCR amplification. Quantitative real-time PCR reaction was incubated in a programmable thermal cycler (iQ5; Bio-Rad, Milan, Italy) starting from 5 min denaturation at 95°C followed by 35 cycles at 95°C for 10 s,

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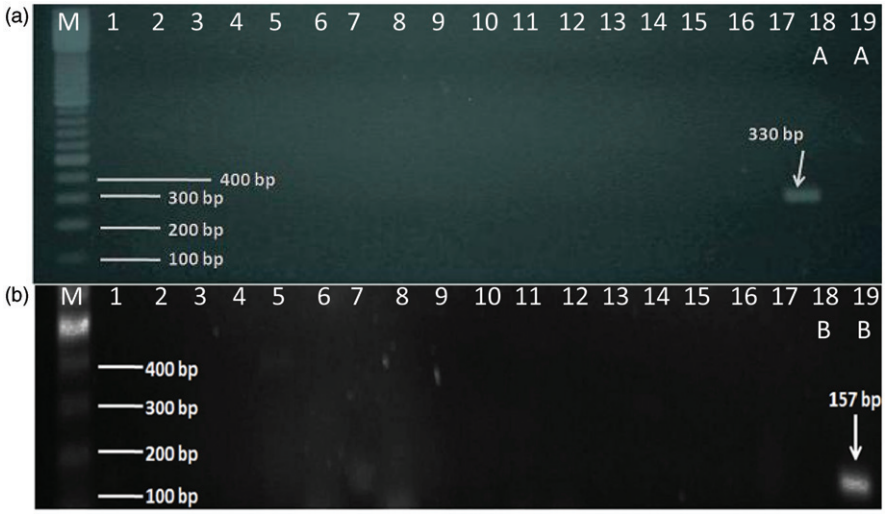

Figure 1. Agarose gel electrophoresis of amplification products from genomic DNA of various fungal isolates obtained with primer pairs ACPKS2 (F/R) specific for *A. carbonarius*, amplifying a fragment of 330 bp (A); primer pairs ANPKS 5/6 specific for *A. niger*, amplifying a fragment of 157 bp. Lanes 1–17: *Alternaria* sp., *Botrytis cinerea*, *Cladosporium* sp., *Cryphonectria parasitica*, *Cylindrocarpon* sp., *Gliocladium* sp., *Cladosporium* sp., *Colletotrichum* sp., *Penicillium* sp., *Phomopsis viticola*, *Sclerotinia sclerotiorum*, *Dothiorella ribis*, *Septoria tritici*, *Trichoderma* sp., *Phoma* sp., *Macrophomina* sp., and *A. niger*, respectively; 18/A, *A. carbonarius*; 19/A, non-template control; 18/B, non-template control; and 19/B, *A. niger*.

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annealing at 62°C for 10 s for *A. carbonarius* and 52°C for *A. niger*, and an extension at 72°C for 15 s. Reactions were performed in 25 µl mixture containing: 12.5 µl of 1 × iQSYBR Green Supermix (Bio-Rad), 10 µM forward and reverse primers, 5 ng of DNA template and deionized sterilized water up to 25 µl. A standard curve was generated from the amplification of ten-fold dilutions of *A. carbonarius* and *A. niger* DNA ranging from 10 to 0.01 ng. These DNA amounts were subject to real-time PCR conjugated with SYBR Green dye using the specific primers ACPKS2 F/R for *A. carbonarius* and ANPKS 5/6 for *A. niger*. The parameter used for data interpretation was the threshold cycle (C_t), which represents the cycle number at which the fluorescence emission exceeds the fixed threshold. Quantification of the unknown DNA samples was carried out by interpolating the samples C_t values with the standard curves (Atoui et al. 2006). At the end of each run, and to ensure that only the specific PCR product was present, a melting curve was performed which was programmed as 81 cycles of 30 s at 55°C.

Results and discussion

Specificity of the PCR reaction

A single fragment of 330 bp of the target DNA was obtained only when genomic DNA from *A. carbonarius* was used with ACPKS2 (F/R) and of 157 bp from *A. niger* with ANPKS (5/6) primers (Figure 1, A and B). The absence of any aspecific band using DNA extracted from other *Aspergillus* spp. and from filaments fungi demonstrates the specificity of primers, and the possibility of the application of quantities using real-time PCR.

Development of the real-time PCR method for A. carbonarius *and* A. niger *DNA quantification*

A standard curve was obtained by plotting the C_t value versus the logarithm of the concentration of each DNA dilution. The linear correlation coefficient of the standard curve was $R^2 = 0.963$ and 0.993 for *A. carbonarius* (Figure 2, A) and *A. niger* (Figure 2, B), respectively, demonstrating the accuracy of PCR-based quantification. Consequently, the use of the generated standard curves allows the quantification of the DNA of *A. carbonarius* and *A. niger* from contaminated table grapevine.

Application of the qPCR method for A. carbonarius *and* A. niger *in artificially contaminated samples*

Application of the qPCR method for *A. carbonarius* and *A. niger* in artificially contaminated samples was performed by interpolating its C_t value against the standard curve. The results were expressed in ng DNA µl^{-1}. The amount of DNA of both fungi did not statistically change until 26 days of post-harvest storage at 4°C, then the quantity of DNA of both fungi significantly increased after 40 days of storage in the cases of Italia and Red Globe but not of Crimson (Figure 3). These results could be partly explained considering the post-harvest ageing

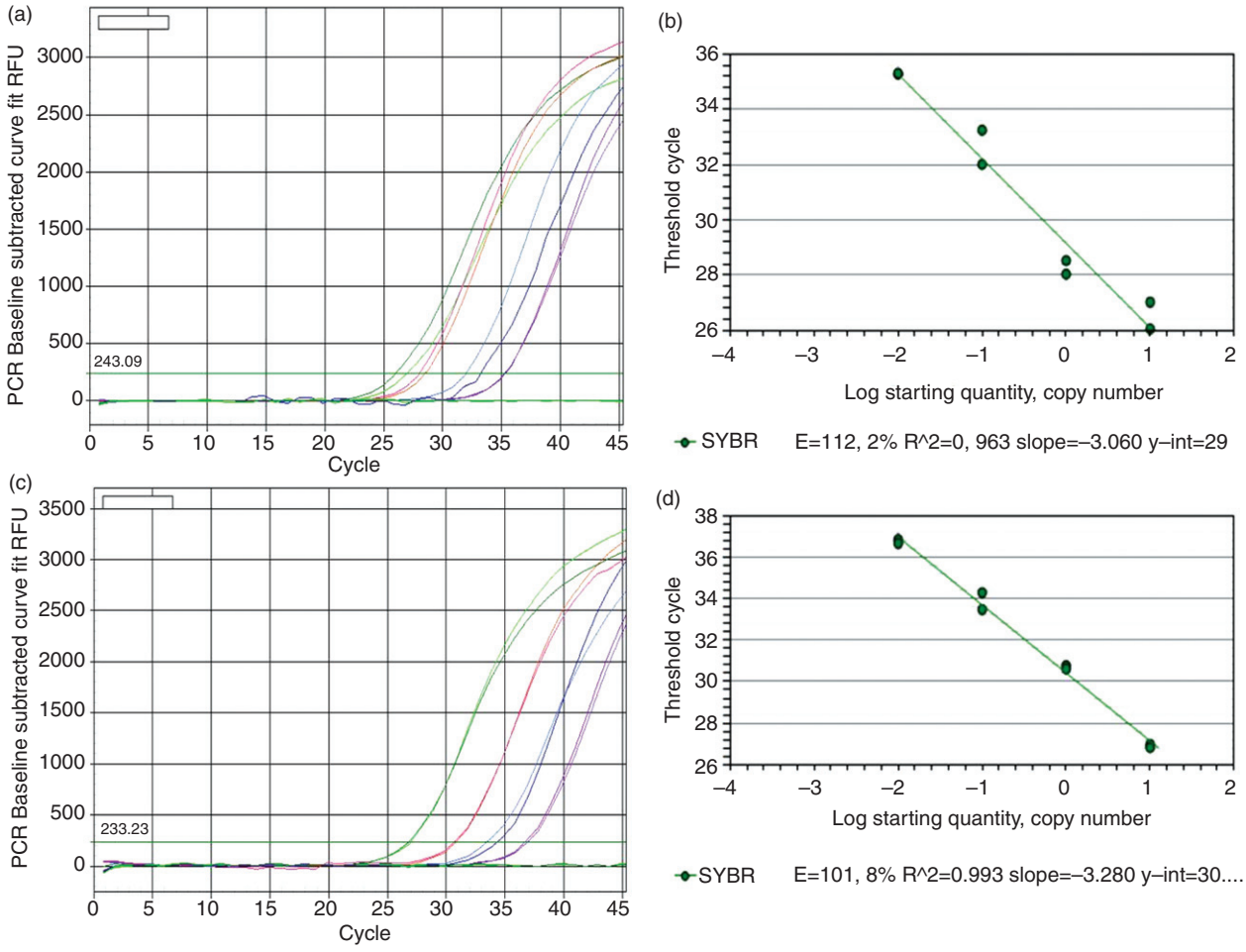

Figure 2. Correlation between log starting quantity of DNA ng μl^{-1} and cycle threshold (C_t) value as assessed by SYBR Green real-time PCR of *A. carbonarius* (A) and *A. niger* (C). The standard curve was generated from the amplification of ten-fold dilutions (from 10 to 0.01 ng μl^{-1}) from target genomic DNA of *A. carbonarius* (B) and *A. niger* (D). The coefficient of linear regression equation of C_t with log DNA concentrations was $R^2 = 0.963$ and 0.993, respectively.

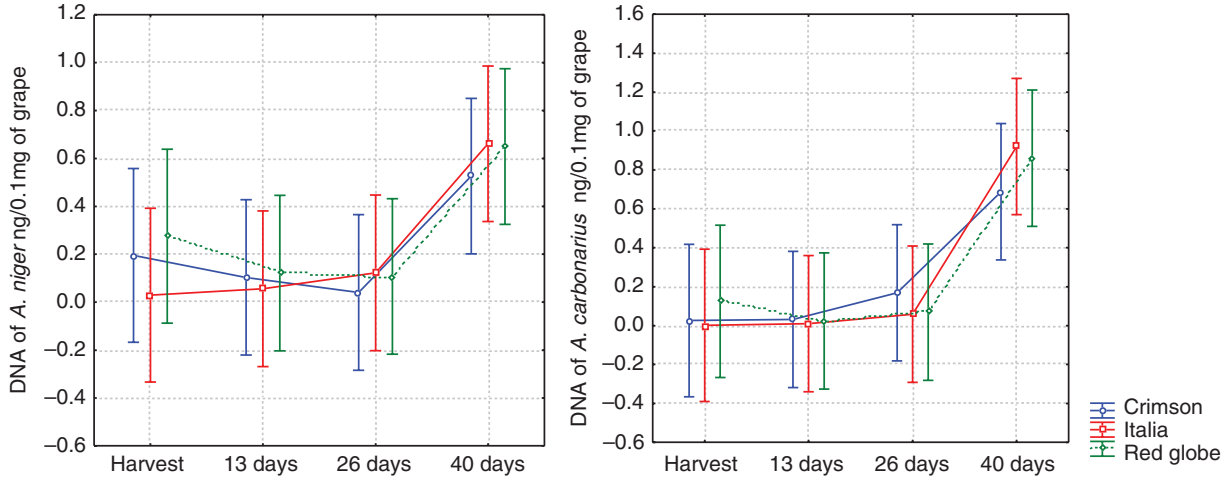

Figure 3. Detection of DNA quantity of *A. carbonarius* and *A. niger* by SYBR Green real-time PCR extracted from inoculated and non-inoculated table grapes of Red globe, Italia, and Crimson seedless, starting from the day of harvest and during 40 days of post-harvest cold storage. Columns report the average of DNA content from four different DNA extractions and amplifications. Bars represent the standard deviation (SD).

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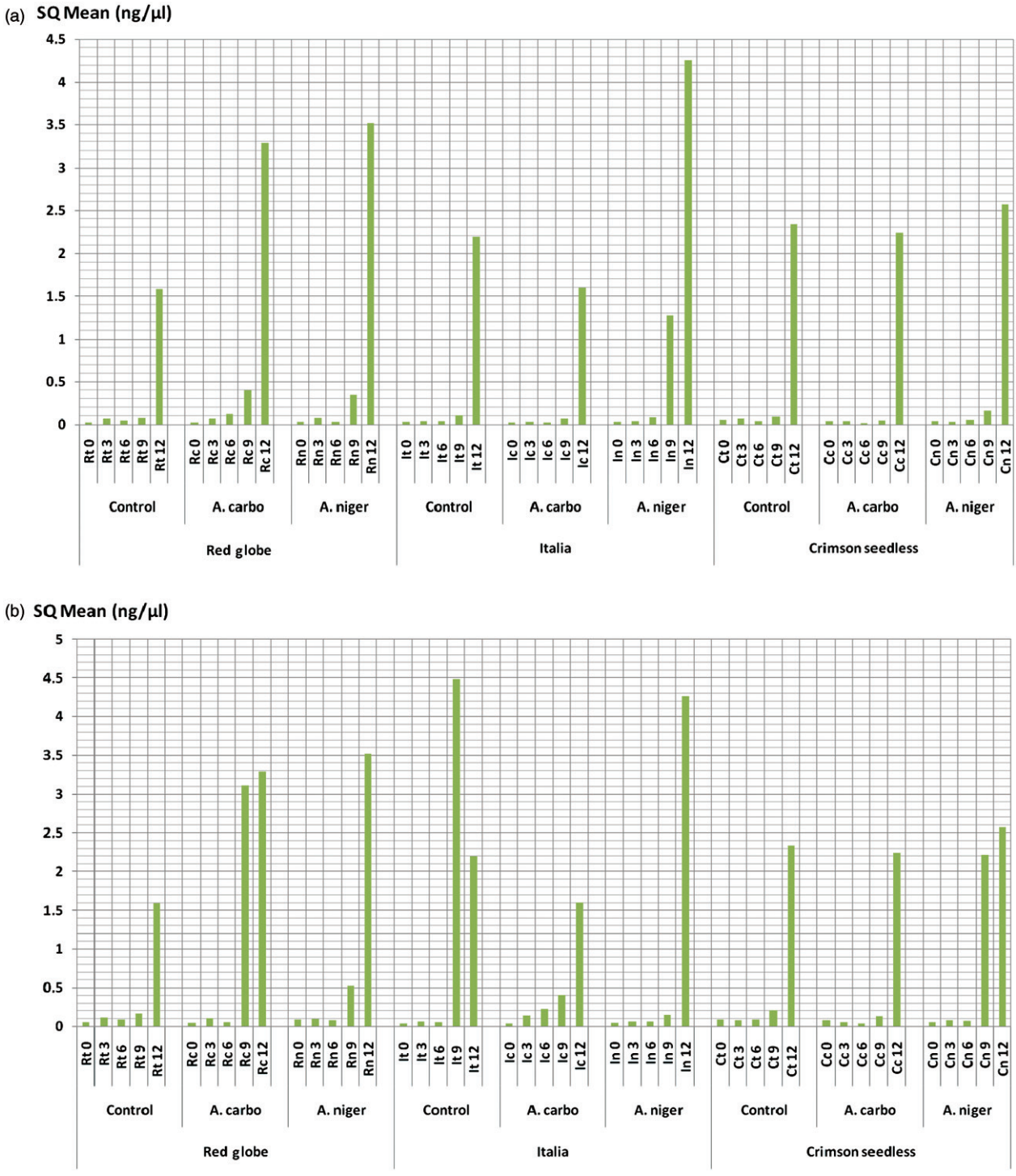

Figure 4. DNA quantification of *A. carbonarius* (A) and *A. niger* (B) by SYBR Green real-time PCR using ACPKS2 (F/R) and ANPKS (5/6) specific primes, respectively, of inoculated and non-inoculated (control) table grapes of Red globe, Italia, and Crimson seedless, during the incubation periods at 30°C (0, 3, 6, 9 and 12 days) after 40 days of post-harvest cold storage.

processes. In fact, during storage time, the grape berries become more susceptible to attack by these fungi. In addition, these results are correlated with those of visual infection, showing a high percentage of infection: up to 70% in berries stored for 40 days then incubated at 30°C for 12 days. Hence, the development of this fungus was very rapid reaching a high percentage of infection after 26 days of post-harvest cold storage. A gradual increase in the DNA content of *A. carbonarius* and *A. niger* in each variety during the incubation periods at 30°C (0, 3, 6, 9 and 12 days) was monitored after 40 days of post-harvest cold storage (Figure 4, A and B). The target fungal DNA, of both *A. carbonarius* and *A. niger*, was detected even

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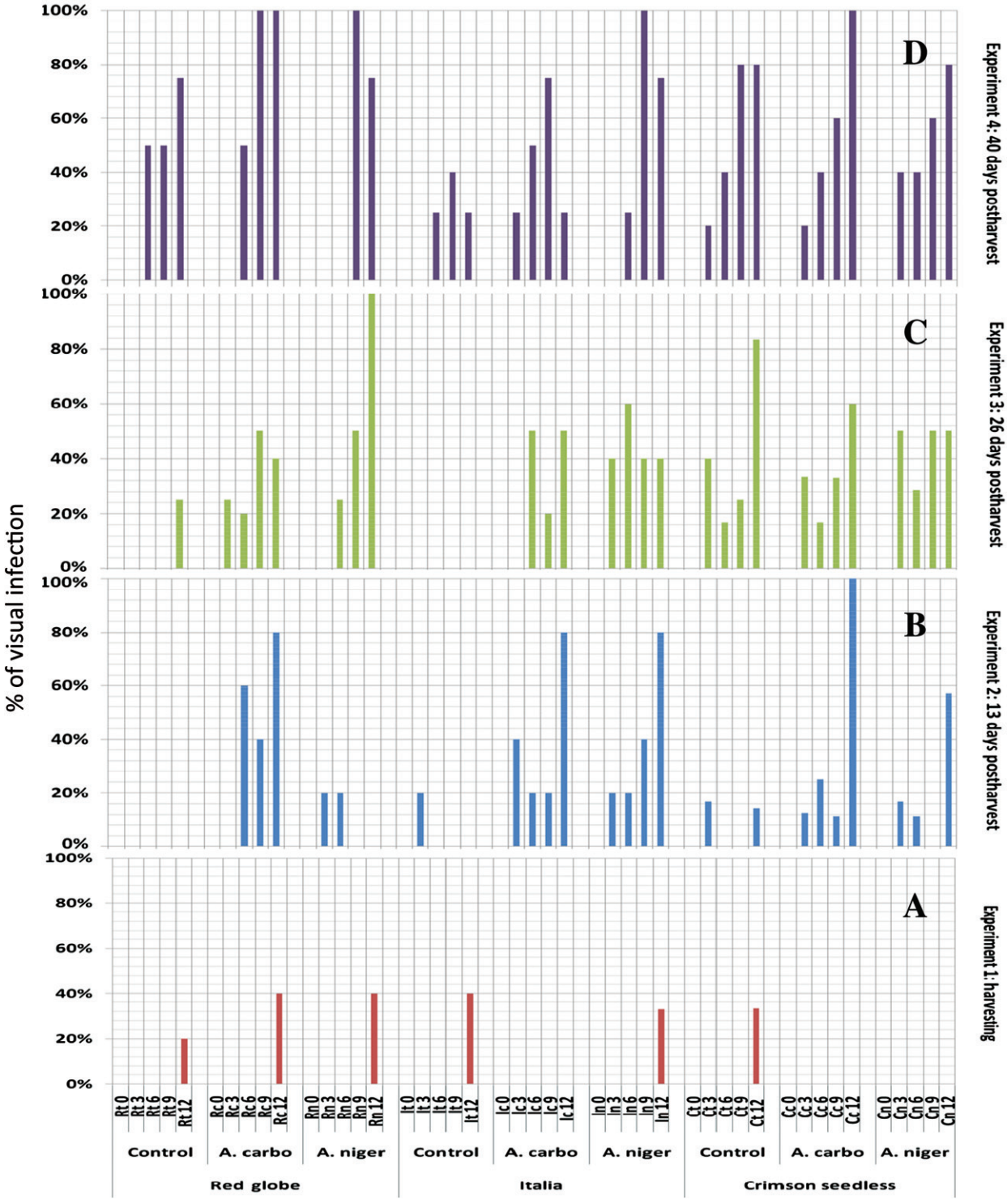

Figure 5. Percentage of infected grapes Red globe (R), Italia (I) and Crimson seedless (C) cultivars with *A. carbonarius* (c) and *A. niger* (n), after 0 (A), 13 (B), 26 (C) and 40 (D) days of post-harvest cold storage during different incubation periods (0, 3, 6, 9 and 12 days) at 30°C. Un-inoculated grapes from each cultivar were used as controls (t).

at time zero (without incubation); the lowest detection limit achieved was $2.37 \times 10^{-2}\,\mathrm{ng\,\mu l^{-1}}$ showing the high sensitivity of the detection method (Figure 4, A and B).

Optimization of a real-time PCR assay is very important, mainly when SYBR Green dye is used. The analysis of the melting curve and the parameters calculated from the standard curve showed that the designed method was highly optimized. The melting curve for *A. carbonarius* showed that only one fragment was amplified and primers did not form other aspecific bands (data not shown).

It was evident that both *A. carbonarius* and *A. niger* were detected in all samples, nevertheless a superficial sterilization was performed. These results correlate with the visual observations (Figure 5); fungal growth was observed not only near the zone of inoculation, but also around the stalk of each berry, where, usually, the fungus starts to develop in non-inoculated samples. Moreover, according to Battilani et al. (2003), *A. carbonarius* as well as *A. niger* can enter berries even without skin damage.

Several amplification methods have been developed with conventional PCR for the analysis of phytopathogenic fungi (competitive PCR) (Singh and Singh 1995; Mahuku and Platt 2002). However, these methods are laborious and insufficiently accurate because the amplification efficiency decreases in later PCR cycles (Ginzinger 2002). An accurate, reliable, and high-throughput quantification of target DNA requires a real-time PCR approach (Lie and Petropoulos 1998; Schmittgen 2001). Real-time PCR quantifies initial concentrations of DNA by the threshold cycle (C_t); the amplification cycle at which the concentration of DNA produced in the PCR reaction gives a visualized fluorescence that climbs above a baseline 'threshold'. Therefore, a lower C_t value corresponds to higher amount of DNA at the beginning of the reaction.

Concentration values obtained by real-time PCR in samples with a starting quantity of 5 ng of total DNA extracted from samples were related to the initial amounts of *A. carbonarius* and *A. niger* DNA. Moreover, the application of the method to quantify these fungi in artificially contaminated matrices was confirmed and detection was executed at longer incubation times at 30°C.

The specificity and high degree of sensitivity of the real-time PCR detection method developed for *A. carbonarius* and *A. niger* provide a good tool for the early detection of this OTA-producing fungi in table grapes and thus prevent OTA entering the food chain. Detection of these fungi, in the case of table grapes, becomes particularly critical around harvest time when OTA production is considered high.

This work described a real-time PCR method that led to the identification and quantification of *Aspergillus carbonarius* and *A. niger* aggregate in three varieties of table grapes without visible symptoms of rotting. This method was rapid and specific with a high sensitivity, providing a useful tool for the early detection of both fungus and the prediction of OTA contamination in table grapes susceptible to be colonized by these species. Moreover, the results obtained could be useful to define a 'quality label' for the assayed grapes in order to improve the safety measures taken to guarantee the production of fresh table grapes.

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References

Ailsa DH, Su-lin LL, Benozir AK, Robert WE, Eileen SS. 2007. Fungi and mycotoxins in vineyards and grape products. Int J Food Microbiol. 119:84–88.

Atoui A. 2006. Approche de la mycotoxinogenese chez Aspergillus ochraceus et *Aspergillus carbonarius*: etudes moleculaire et physiologique [PhD thesis]. [Tolouse (France)]: ENSAT.

Atoui A, Mathieu F, Lebrihi A. 2006. Targeting a polyketide synthase gene for *Aspergillus carbonarius* quantification and ochratoxin A assessment in grapes using real-time PCR. Int J Food Microbiol. 115:313–318.

Battaglia R, Hatzold T, Kroes R. 1996. Occurrence and significance of ochratoxin A in food. Food Addit Contam. 13:1–3.

Battilani P, Pietri A, Bertuzzi T, Languasco L, Giorni P, Kozakiewicz Z. 2003. Occurrence of ochratoxin A-producing fungi in grapes grown in Italy. J Food Protect. 66:633–636.

Battilani P, Logrieco A, Giorni P, Cozzi G, Bertuzzi T, Pietri A. 2004. Ochratoxin A production by *Aspergillus carbonarius* on some grape varieties grown in Italy. J Sci Food Agricult. 84(13):1736–1740.

Bellì N. 2006. Ochratoxin A and ochratoxigenic moulds in grapes, must and wine. Ecophysiological studies [PhD thesis]. [Lleida (Spain)]: Universitat de Lleida.

Bluhm BH, Flaherty JE, Cousin MA, Woloshuk CP. 2002. Multiplex polymerase chain reaction assay for the differential detection of trichothecene- and fumonisin-producing species of Fusarium in cornmeal. J Food Protect. 65:1955–1961.

Ginzinger DG. 2002. Gene quantification using real-time quantitative PCR: an emerging technology hits the main stream. Exp Hematol. 30:503–512.

González-Salgado A, Patiño B, Gil-Serna J, Vázquez C, González-Jaén MT. 2009. Specific detection of *Aspergillus carbonarius* by SYBR Green and TaqMans quantitative PCR assays based on the multicopy ITS2 region of the rRNA gene. FEMS Microbiol Lett. 295:57–66.

Guzev L, Danshin A, Ziv S, Lichter A. 2006. Occurrence of ochratoxin A producing fungi in wine and table grapes in Israel. Int J Food Microbiol. 111:S67–S71.

Leong SL, Hocking AD, Scott ES. 2006. Survival and growth of *Aspergillus carbonarius* on wine grapes before harvest. Int J Food Microbiol. 111(1):S83–S87.

Lie YS, Petropoulos CJ. 1998. Advances in quantitative PCR technology: 5′ nuclease assays. Curr Opin Biotechnol. 9:43–48.

Mahuku GS, Platt HW. 2002. Quantifying *Verticillium dahliae* in soils collected from potato fields using a competitive PCR assay. Am J Potato Res. 97:107–117.

Parenicova L, Skouboe P, Samson RA, Rossen L, Visser J. 2000. Integration of modern taxonomic methods of *Penicillium* and *Aspergillus* classification. In: Samson RA, Pitt JI, editors. Molecular tools for the classification

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of black Aspergilli. Amsterdam (the Netherlands): Hardwood, p. 413–424.

Patiño B, González-Salgado A, González-Jaeń MT, Vázquez C. 2005. PCR detection assays for the ochratoxin-producing *Aspergillus carbonarius* and *Aspergillus ochraceus* species. Int J Food Microbiol. 104: 207–214.

Pitt JI, Hocking AD. 1996. Current knowledge of fungi and mycotoxins associated with food commodities in Southeast Asia. Paper presented at: 17th Asean Technical Seminar on Grain Postharvest Technology – Mycotoxin Contamination in Grains; Lumut (Malaysia), Book Series: Aciar Technical Reports No. 37. p. 5–10.

Raper KB, Fennell DI. 1965. The genus *Aspergillus*. New York, NY: Williams & Wilkins.

Schmittgen TD. 2001. Real-time quantitative PCR. Methods. 25:383–385.

Singh RP, Singh US. 1995. Polymerase chain reaction technology in plant pathology. In: Hadidi A, Levy L, Podleckis EV, editors. Molecular methods in plant pathology. London (UK): CRC Press, p. 167–187.

Taylor JW, Geiser DM, Burt A, Koufopanou V. 1999. The evolutionary biology and population genetics underlying fungal strain typing. Clinical Microbiol Rev. 12:126–146.

Van der Merwe KJ, Steyn PS, Fourie L. 1965. Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus*. Wilh., Nature. 265:1112–1113.

Varga J, Kozakiewicz Z. 2006. Ochratoxin A in grapes and grape derived products. Trends Food Sci Technol. 17:72–81.

Yaseen T, Ayoub F, Ricelli A. 2009. Partial polyketide synthases sequence of *A. niger* and specific PCR based detection. Paper presented at: ISM Conference 2009. Proceedings of the ISM Conference Tulln/Vienna; Austria.

Yaseen T, Ippolito A, Faretra F, Casulli F. 2004. Molecular diagnosis and biological control of Phytophthora-citrus root rot [PhD thesis]. [Bari (Italy)]: University of Bari.

Zhao J, Kong F, Li R, Wang X, Wan Z, Wang D. 2001. Identification of *Aspergillus fumigatus* and related species by nested PCR targeting ribosomal DNA internal transcribed spacer regions. J Clinical Microbiol. 39:2261–2266.

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