

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

**Specific PCR primers for the detection of isolates of *Aspergillus carbonarius* producing ochratoxin A on grapevine.**

**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/93064> since

*Terms of use:*

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)



UNIVERSITÀ DEGLI STUDI DI TORINO

1  
2  
3  
4  
5  
6  
7

*The final publication is available at Springer via <http://dx.doi.org/10.1007/s13213-010-0131-3>*

8 **Specific PCR primers for the detection of isolates of *Aspergillus carbonarius* producing**  
9 **ochratoxin A on grapevine**

10

11 Davide Spadaro<sup>1,2\*</sup>, Subban Patharajan<sup>1</sup>, Muthusamy Kartikeyan<sup>1</sup>, Alessia Lorè<sup>1</sup>, Angelo Garibaldi<sup>1</sup>,  
12 Maria Lodovica Gullino<sup>1</sup>

13

14 <sup>1</sup>Centre of Competence for the Innovation in the Agro-environmental Sector, Università degli Studi di  
15 Torino, via L. da Vinci 44, I-10095 Grugliasco (TO), Italy; <sup>2</sup>DiVaPRA - Plant Pathology, Università  
16 degli Studi di Torino, via L. da Vinci 44, I-10095 Grugliasco (TO), Italy.

17

18 **Abstract**

19 *Aspergillus carbonarius* is the main fungus responsible of ochratoxin A (OTA) production on grapes  
20 and wine. Fungal polyketide synthases are involved in OTA biosynthesis in *Aspergillus* species. The  
21 ketosynthase (KS) domain of a polyketide synthase (*pks*) gene of *A. carbonarius* was isolated, cloned  
22 and sequenced. The nucleotide sequence showed high similarity to the KS domain of other *Aspergillus*  
23 species. The sequence was used to design a new set of primers in order to identify isolates of *A.*  
24 *carbonarius* potential producers of OTA. The primers specifically amplified all the *A. carbonarius*  
25 strains tested and did not amplify other species of *Aspergillus* and *Penicillium*, normally found on  
26 grapes or involved in OTA biosynthesis. Further, the gene expression was related to the OTA  
27 production. Both the gene transcription and the mycotoxin were higher when *A. carbonarius* was  
28 grown on YES at 15°C and 30°C, whereas a low transcription and mycotoxin presence were observed  
29 when the fungus was grown on PDB at the same temperatures. No transcription and OTA production

---

\* Corresponding author: Phone: +39-011-6708942, Fax: +39-011-6709307,  
Email: [davide.spadaro@unito.it](mailto:davide.spadaro@unito.it)

30 were observed when the fungus was grown on YES or PDB at 10°C. The primers will be useful for the  
31 detection of ochratoxigenic strains of *A. carbonarius* both in vineyard and during wine production.

32

33 **Keywords:** *Aspergillus carbonarius*; HPLC; RT-PCR; ochratoxin A; polyketide synthase; vineyard.

34

## 35 **Introduction**

36

37 Ochratoxin A (OTA) has been reported as a dangerous nephrotoxic and carcinogenic mycotoxin,  
38 produced by various species of *Aspergillus* and *Penicillium* (Petzinger and Ziegler, 2000). The  
39 presence of OTA in wine was reported for the first time by Zimmerli and Dick (1996). Since then,  
40 several surveys on wines have shown a considerable level of contamination, with higher toxin  
41 concentrations and incidence rate in red wines originating from southern and warmer regions of Europe  
42 (Pietri et al., 2001) and lower contamination levels in northern and cooler regions (Spadaro et al.  
43 2010a). OTA has also been detected in other grape-derived products (Battilani et al., 2004). OTA  
44 contamination on grapes takes place in the field and is produced mainly by black aspergilli. *Aspergillus*  
45 *carbonarius* is the most important OTA-producing species.

46 To prevent OTA contamination in grapes or in any other foodstuffs, it is necessary to have a rapid and  
47 specific method to detect OTA producing fungi in early stages of infection. Detection of these fungi, in  
48 the case of grapes, is particularly critical around harvest time, when contamination levels and OTA  
49 production are considered high. Usual identification and quantification methods of food-borne fungi  
50 require multiple steps. They are time-consuming and often, mycological expertise is necessary.  
51 Different molecular techniques can be used to identify the presence of *A. carbonarius* in vineyard,  
52 including sequencing of amplified products or PCR-RFLP (Gullino et al., 2008). Nowadays, many  
53 studies have been reported based on PCR methods to produce more specific, sensitive, and rapid  
54 detection of the target organism. One of the most important factors in the development of such methods  
55 is the reliability of the primer set designed and the targeted DNA sequence of interest organism.

56 Chemically, OTA is composed of an isocoumarin polyketide and the (modified) amino acid  
57 phenylalanine, which are linked via peptide bond. Fungal polyketide synthases (PKSs) have recently  
58 been demonstrated to be involved in OTA biosynthesis in both *Penicillium* and *Aspergillus* species

59 (O'Callaghan et al., 2003; Geisen et al., 2004). PKSs are multifunctional proteins with several domains  
60 encoded by one gene. The nucleotide sequences of the domains are homologous between different  
61 fungal species (Bingle et al., 1999). In the present study, we designed a set of specific primers, AcPKS-  
62 F1 (forward) and AcPKS-R1 (reverse) on the DNA sequence of a putative polyketide synthase (*pks*)  
63 gene of *A. carbonarius* strain AC06. This primer set was tested for primer specificity by PCR on  
64 different species of *Aspergillus* and *Penicillium*, normally present in vineyard or able to produce  
65 ochratoxin A. Further, reverse transcription studies were performed to confirm the involvement of the  
66 *pks* gene amplified during OTA biosynthesis. The *pks* gene was expressed only under OTA permissive  
67 conditions.

68

## 69 **Materials and methods**

70

### 71 **Fungal strain and culture conditions**

72 *Aspergillus carbonarius* strain AC06 (Agroinnova culture collection centre, University of Turin, Italy),  
73 an ochratoxin A producing strain isolated from vineyard in Liguria region of Italy, was used throughout  
74 this study. identified by using a polyphasic approach (Oliveri et al., 2008) based on ITS sequencing,  
75 calmodulin sequencing, and PCR-RFLP (Spadaro et al., 2009). The ITS sequences of the strain was  
76 deposited at the National Centre for Biotechnology Information (NCBI, GenBank, New York, USA)  
77 with the following accession number: GQ468224 (Spadaro et al. 2010b). AC06 was routinely grown at  
78 25°C on potato dextrose agar (PDA) (Merck) for 5 days. Conidial suspensions ( $1 \times 10^6$  spores  $\text{ml}^{-1}$ ) were  
79 inoculated into 250 ml Erlenmeyer flasks containing 100 ml of YES (30g  $\text{l}^{-1}$  yeast extract, 200 g  $\text{l}^{-1}$   
80 sucrose, 0.5 g  $\text{l}^{-1}$  magnesium sulphate) and PDB [(24 g  $\text{l}^{-1}$ ) Merck, Darmstadt, Germany] and incubated  
81 at 15°C and 27°C for 9 days. After incubation, the mycelium was harvested by filtration, frozen in

82 liquid nitrogen and stored at -80°C for nucleic acid extraction. The filtered PDB and YES liquid broth  
83 were analysed for OTA content.

84 The other strains, tested for primer specificity, were several strains of *A. carbonarius* isolated from  
85 vineyards in Northern Italy, or purchased at the Centralbureau voor Schimmelcultures (CBS), Utrecht,  
86 The Netherlands. The strains purchased at the CBS were *A. carbonarius* CBS127.49, *Aspergillus*  
87 *ellipticus* CBS707.79, *Aspergillus tubingensis* CBS119556, *Aspergillus niger* CBS119557, *Aspergillus*  
88 *aculeatus* CBS116.80, *Aspergillus japonicus* CBS119560, *Aspergillus brasiliensis* CBS121619,  
89 *Aspergillus ochraceus* CBS132.52, *Penicillium nordicum* CBS110769, and *Penicillium verrucosum*  
90 CBS226.71.

91

#### 92 **DNA extraction, PCR, cloning and sequencing**

93 The frozen fungal mycelium was ground to fine powder, and 100 mg powder were taken to extract the  
94 DNA, by using the NucleoSpin Plant DNA kit (Macherey-Nagel, Iuren, Germany) according to the  
95 manufacturer's instructions. PCR was performed on the genomic DNA to amplify the ketosynthase  
96 domain of polyketide synthase using the KS1 (GGRTCNCCLARYTGIGTICCGTICCRTGIG) and  
97 KS2 (MGIGARGCIYTIGCIATGGAYCCICARCA) primers. The amplified product was excised from  
98 the gel and purified using the S.N.A.P. Gel purification system (Invitrogen Life Technologies,  
99 Corlsbad, CA, USA). The resulting DNA was cloned onto pCR2.1 TOPO plasmid vector (Invitrogen  
100 Life Technologies, USA). The recombinant plasmid was purified using the rapid plasmid system and  
101 the insert was sequenced by BMR genomics DNA sequencing service Centre (Padova, Italy) with the  
102 ABI PRISM 3730 XL DNA sequencer. Sequencing data were used to design the primer pair AcPKS-F1  
103 (AGCATCTATGCTGGCCAATC) and AcPKS-R1 (AATGTACTCTCGCGGGCTAA) from two  
104 highly conserved regions. PCR conditions were standardised and tested for specificity on the DNA of  
105 different species of *Aspergillus* and *Penicillium*. PCR consisted of 100-200 ng DNA, 50 mM KCl, 10

106 mM Tris-HCl, 80  $\mu$ M each dNTP, 1  $\mu$ M each primer, 2 mM MgCl<sub>2</sub>, and 1 U DNA polymerase. A *T-*  
107 *gradient*<sup>®</sup> thermal cycler (Biometra, Germany) was programmed as follows: an initial step at 94°C for 4  
108 min, followed by 35 cycles at 94°C for 40 s, 55°C for 40 s, 72°C for 40 s, and a final elongation step of  
109 72°C for 10 min.

110

### 111 **RNA extraction and cDNA synthesis**

112 Mycelia were harvested from liquid culture, frozen immediately at -80°C, and then ground in liquid  
113 nitrogen with sterilized pre-cooled mortar and pestle. Total RNA was extracted from the pulverised  
114 mycelium using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The  
115 extracted RNA was treated with RNase-free DnaseI (Promega, Madison, WI) to eliminate traces of  
116 contaminating DNA. The cDNA was synthesised using about 1.5  $\mu$ g of total RNA, oligo (dT)<sub>18</sub> primer,  
117 and the SuperScript III reverse transcriptase (Invitrogen, San Diego, CA) according to the  
118 manufacturer's protocol. The cDNA was stored at 4°C.

119

### 120 **RT-PCR**

121 For the analysis of *AcPks* gene expression in two different media with permissive and restrictive  
122 conditions, aliquots of cDNA were used as templates for RT-PCR amplification with the primers  
123 AcPKS-F1 and AcPKS-R1. The number of cycles used was set to 30 cycles, to avoid amplification  
124 reaching a plateau and to allow a better normalization of the template amounts. RT-PCR conditions  
125 were 94°C for 5 min, 30 cycles at 94°C for 30 s, 55°C for 50 s, 72°C for 50 s, and a final extension step  
126 at 72°C for 5 min.

127

### 128 **Extraction of OTA from culture broth**

129 For the detection of OTA levels in the media, the culture filtrate (3 ml) was centrifuged at 10,000 rpm  
130 for 10 min. The samples were diluted ten times with distilled water, and OTA was extracted from the  
131 culture filtrate using and quantified through HPLC by following the method of Bragulat et al. (2001).  
132 Samples were analyzed in a HPLC Agilent series 1100 formed by a degasser, an autosampler, a  
133 quaternary pump, a thermostated column and a fluorimeter. An analytical column RP-18 (150 mm x  
134 4.6 mm i.d., 5  $\mu$ m) with a pre-column was used. The mobile phase, eluting at 1 ml min<sup>-1</sup>, consisted of  
135 an isocratic mixture of acetonitrile:water:acetic acid (45:45:10) for 18 min. Samples of 100  $\mu$ l were  
136 injected onto the HPLC column and the retention time of OTA was 6.15 min. The amount of OTA in  
137 the final solution was determined by using a calibration graph of concentration versus peak area and  
138 expressed as ng ml<sup>-1</sup>, achieved by injection onto the HPLC column of 100  $\mu$ l of standard solutions of  
139 OTA (Sigma Chemical Co.). The standard solutions had concentrations of 0.5, 1.0, 5.0, 10.0, 25.0,  
140 50.0, and 100.0  $\mu$ l l<sup>-1</sup>. The recovery was determined on a blank YES broth spiked at three  
141 concentrations of OTA (0.1, 2.0 and 10.0 ng ml<sup>-1</sup>). Recovery was performed in four replicates and  
142 ranged from 90.8% to 92.1%. Repeatability ranged from 2.64 to 2.71 % per replicate. The detection  
143 limit was 0.01 ng OTA ml<sup>-1</sup> YES medium.

144

## 145 **Results and Discussions**

146

### 147 **Primer specificity**

148 The PCR performed with the primers KS1 and KS2 on the genomic DNA of *A. carbonarius* AC06  
149 permitted to amplify the ketosynthase domain of a putative polyketide synthase, called *AcPks* and  
150 deposited in GenBank with the accessions number EU926157. An open reading frame (ORF) of 569 bp  
151 encoding a 190 amino acid protein was characterized (GenBank: ACH47947.1). Protein BLAST  
152 revealed that the several sequences had a query coverage of 99 or 100%. The putative protein sequence

153 belongs to the ketoacylsynthase superfamily, and in particular to the polyketide synthases.  
154 Specifically, *Acps* displayed nucleotide identity (99%) to the PKS sequence of a *A. carbonarius*  
155 (Accession no. AAS98197.1), and lower level of nucleotide identity (84%) to a protein of *Aspergillus*  
156 *niger* strain CBS 513.88 (XP001394930.1) and to a putative PKS of *Aspergillus clavatus* strain NRRL  
157 1 (XP001271445.1). The alignment showed high homology with two main conserved regions in the  
158 ketosynthase domain of *Aspergillus* spp.. A new set of primers (AcPKS-F1/AcPKS-R1) was designed  
159 on the conserved regions and the primers amplified a PCR product with an expected size of 186 bp  
160 (Fig. 1). The primers were tested for their specificity against several strains of *A. carbonarius* isolated  
161 from Italian vineyards and another strain of *A. carbonarius* from the CBS. All the *A. carbonarius*  
162 isolates amplified a band of the expected size (Fig. 1). Further, the primers were tested for their  
163 specificity on the genomic DNA of different species of *Aspergillus* and *Penicillium* isolated from  
164 Italian vineyards or coming from the CBS. The species tested as negative control include species of  
165 *Aspergillus* known from literature to be ochratoxigenic (*A. ochraceus*, belonging to the section  
166 *Circumdati* group) or belonging to the section *Nigri*. The number of reports dealing with the production  
167 of OTA by members of section *Nigri* has been increasing since the first description of OTA production  
168 by *Aspergillus niger* var. *niger* (Abarca et al. 1994). *Aspergillus* section *Nigri* includes uniseriate  
169 species (*A. aculeatus* and *A. japonicus*), the species *A. carbonarius*, species belonging to *A. niger*  
170 aggregate (*A. tubingensis*, *A. niger*, *A. brasiliensis*) and uncommon species, such as *A. ellipticus*.  
171 Some isolates of the species *A. niger*, *A. carbonarius* (Horie, 1995), *A. japonicus* (Dalcero et al., 2002),  
172 *A. aculeatus* (Battilani et al., 2003), and *A. tubingensis* (Perrone et al., 2007) can be ochratoxin A  
173 producers. Moreover, also two species of *Penicillium*, known to produce ochratoxin A, were included:  
174 *P. verrucosum* occurs predominantly in stored cereals in temperate regions, while *P. nordicum* can  
175 contaminate protein-rich substrates, mainly meat (Larsen et al. 2001). The PCR results showed that a  
176 unique PCR product was amplified only from the genomic DNA of *A. carbonarius* isolates. No

177 amplification was observed from the genomic DNA of the other species of *Aspergillus* or *Penicillium*  
178 tested (Fig. 1). The results showed that only the strains belonging to *A. carbonarius* species produced a  
179 PCR amplification. A single PCR product observed from each DNA of the ochratoxigenic isolates of *A.*  
180 *carbonarius* confirmed the specificity of the primers.

181 The primer sets were selected from the most conserved regions of the ketosynthase domain of a  
182 polyketide synthase, a key gene required for OTA production. Probably, these DNA conserved regions  
183 were not present in the other fungal species tested, although many of them are known as producers of  
184 ochratoxins or other polyketides. The PCR amplification is often used for the detection of pathogenic  
185 or toxigenic microorganisms in various substrates, including foods (Dao et al., 2005). Two PCR assays  
186 were developed to detect *A. carbonarius* and *A. ochraceus* with primers designed on the internal  
187 transcribed spacers of rDNA units (Patiño et al., 2005). Several reports demonstrated that the primers  
188 for the detection of mycotoxin producing fungi in foods should be designed on the conserved domains  
189 of genes involved in the biosynthetic pathway of mycotoxins (O'Callaghan et al., 2003; Dao et al.,  
190 2005). When the primers are designed on other genes, such as calmodulin, not involved in mycotoxin  
191 biosynthesis, the PCR amplification can help to differentiate the species (Perrone et al., 2004) but it  
192 cannot be used to identify mycotoxigenic isolates (Mulè et al., 2006). Polyketosynthases (PKSs) are  
193 multifunctional enzymes which are encoded by a single gene and typically possess up to eight types of  
194 functional domains (Bingle et al., 1999). The cloning and molecular characterization of many genes  
195 encoding fungal PKSs has been greatly facilitated by the conserved nature of these functional domains,  
196 which has allowed to design gene probes and degenerated primers used to isolate the gene fragments  
197 encoding the PKS domains in several fungal species (Cox et al., 2004; Nicholson et al., 2001). This  
198 approach has been successfully employed to characterize five KS domains belonging to putative PKSs  
199 in *A. carbonarius* (Atoui et al., 2006). Previous PCR approaches for the quantification of *A.*  
200 *carbonarius* isolates were developed on PKS domains: a SYBR-Green I approach targeted to the

201 acyltransferase domain of a PKS (Atoui et al., 2007). Also Selma et al. (2008) developed a real-time  
202 procedure based on two specific primers directed to the ketosynthase domain of a polyketide synthase  
203 for the detection of *A. carbonarius* in wine grapes, but they did not confirm the correlation of the gene  
204 expression with the OTA production.

205

### 206 **Transcription of *pks* gene and ochratoxin A production**

207 In order to determine if a positive correlation existed between the cloned KS domain of the *pks* gene  
208 identified in this study and OTA production, the strain AC06 of *A. carbonarius* was grown in two  
209 media incubated at different temperatures known to affect OTA biosynthesis (Spadaro et al., 2010b).  
210 The transcription of the *pks* gene was monitored using a reverse transcription (RT)-PCR based  
211 approach, and contemporarily OTA production was measured through HPLC analysis. When the strain  
212 AC06 was grown in YES (permissive) medium for 9 days, it showed higher production of OTA ( $2.9 \mu\text{g}$   
213  $\text{l}^{-1}$ ) at 30°C, while OTA level was lower when the strain was incubated at 15°C ( $1.8 \mu\text{g} \text{l}^{-1}$ ). Similarly,  
214 when grown in PDB (restrictive) medium for 9 days, AC06 produced higher amounts of OTA at 30°C  
215 ( $0.5 \mu\text{g} \text{l}^{-1}$ ) than at 15°C ( $0.3 \mu\text{g} \text{l}^{-1}$ ), but the level of OTA produced was significantly lower on PDB  
216 than on YES. Less production of OTA by *A. ochraceus* on PDB was reported also by O'Callaghan et  
217 al. (2006). No OTA production was observed when the strain was incubated at 10°C either in YES or  
218 PDB (Fig. 2). Several researchers reported that the optimum temperature for growth and OTA  
219 production of *A. carbonarius* strains is from 25°C to 35°C (Mitchell et al., 2004; Battilani, et al., 2004;  
220 Bellí et al., 2004a,b; Esteban et al., 2004). Analysis of the transcript level clearly indicated that the  
221 *AcPks* gene was expressed differentially in different environmental conditions (media and temperature  
222 of growth) and its transcription was related to the level of OTA production (Fig. 3). Temperature of  
223 10°C did not affect significantly the expression level in both media.

224 A similar approach was employed to characterize five KS domains belonging to putative *pks* in *A.*  
225 *carbonarius* (Atuoi et al., 2006). More recently, also Gallo et al. (2009) reported that a *AcPks* gene  
226 from *A. carbonarius* was differentially expressed on permissive and restrictive media and it was related  
227 to the level of OTA production. The same authors reported that *AcPks* gene was differentially  
228 expressed according to the pH of the medium. Higher pH values were accompanied by a reduction of  
229 the *pks* gene transcript accumulation and by a reduced level of OTA production (Gallo et al., 2009).  
230 Geisen (2004) reported that the expression of *otapksPN* in *Penicillium nordicum* was down regulated  
231 under acidic conditions which accompanied the decrease in OTA biosynthesis.  
232 The results obtained in the current study revealed that the PCR primers developed will be useful to  
233 detect specifically OTA producing strains of *A. carbonarius* both in vineyard or in wine industries.

234

#### 235 **Acknowledgments**

236 This research was carried out partly with a grant from the Piedmont Region for the project "Evaluation  
237 of the risk, prevention and management of the presence of mycotoxins in the wine production chain of  
238 Piedmont" and partly with a grant from the Fondazione Cassa di Risparmio di Torino "Evaluation and  
239 prevention of the risk of ochratoxin A in the Piemontese wine chain" to AGROINNOVA. Moreover,  
240 Subban Patharajan and Muthusamy Karthikeyan acknowledge the Italian Ministry for University and  
241 Research and the Compagnia di San Paolo for the PostDoc grants "Bando per borse a favore di giovani  
242 ricercatori indiani".

243

#### 244 **References**

245 Abarca ML, Bragulat MR, Castellá G, Cabañes FJ (1994) Ochratoxin A production by strains of  
246 *Aspergillus niger* var. *niger*. *Appl Environ Microbiol* 60:2650-2652

247 Atoui A, Mathieu F, Lebrihi A (2007) Targeting a polyketide synthase gene for *Aspergillus*  
248 *carbonarius* quantification and ochratoxin A assessment in grapes using real-time PCR. Int J Food  
249 Microbiol 115:313–318

250 Atoui A, Dao HP, Mathieu F, Lebrihi A (2006) Amplification and diversity analysis of ketosynthase  
251 domains of putative polyketide synthase genes in *Aspergillus ochraceus* and *Aspergillus*  
252 *carbonarius* producers of ochratoxin A. Mol Nutr Food Res 50:488-493

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

255 Battilani P, Pietri A, Logrieco A (2004) Risk assessment and management in practice: ochratoxin in  
256 grapes and wine. In: Magan N, Olsen M (eds) Mycotoxins in food: detection and control,  
257 Woodhead, Cambridge, pp 244-261

258 Bellí N, Marín S, Sanchis V, Ramos AJ (2004a) Influence of water activity and temperature on growth  
259 of isolates of *Aspergillus* section *Nigri* obtained from grapes. Int J Food Microbiol 96:19-27

260 Bellí N, Ramos AJ, Sanchis V, Marín S (2004b) Incubation time and water activity effects on  
261 ochratoxin A production by *Aspergillus* section *Nigri* strains isolated from grapes. Lett Appl  
262 Microbiol 38:72-77

263 Bingle LEH, Simpson TJ, Lazarus CM (1999) Ketosynthase domain probes identify two subclasses of  
264 fungal polyketide synthase genes. Fungal Genet Biol 26:209-223

265 Bragulat MR, Abarca ML, Cabañes FJ (2001) An easy screening method for fungi producing  
266 ochratoxin A in pure culture. Int J Food Microbiol 71: 139-144

267 Cox RJ, Glod F, Hurley D, Lazarus CM, Nicholson TP, Rudd BAM, Simpson TJ, Wilkinson B, Zhang  
268 Y (2004) Rapid cloning and expression of a fungal polyketide synthase gene involved in  
269 squalestatin biosynthesis. Chem Commun 20:2260-2261

270 Dalcero A, Magnoli C, Hallak C, Chiacchiera SM, Palacio G, Rosa CAR (2002) Detection of  
271 ochratoxin A in animal feeds and capacity to produce this mycotoxin by *Aspergillus* section *Nigri* in  
272 Argentina. *Food Addit Contam* 19:1065–1072

273 Dao HP, Mathieu F, Lebrihi A (2005) Two primer pairs to detect OTA producers by PCR method. *Int J*  
274 *Food Microbiol* 104:61-67

275 Esteban A, Abarca ML, Bragulat MR, Cabañes FJ (2004) Effects of temperature and incubation time  
276 on production of ochratoxin A by black aspergilli. *Research Microbiol* 155:861-866

277 Gallo A, Perrone G, Solfrizzo M, Epifani F, Abbas A, Dobson ADW, Mulé G (2009) Characterization  
278 of a *pks* gene which is expressed during ochratoxin A production by *Aspergillus carbonarius*. *Int J*  
279 *Food Microbiol* 129:8-15

280 Geisen R (2004) Molecular monitoring of environmental conditions influencing the induction of  
281 ochratoxin A biosynthesis genes in *Penicillium nordicum*. *Mol Nutr Food Res* 48:532-540

282 Geisen R, Mayer Z, Karolewicz A, Farber P (2004) Development of a real time PCR system for  
283 detection of *Penicillium nordicum* and for monitoring ochratoxin A production in foods by targeting  
284 the ochratoxin polyketide synthase gene. *System Appl Microbiol* 27:501-507

285 Gullino ML, Lorè A, Muthusamy K, Spadaro D, Garibaldi A (2008) Monitoring of ochratoxin-  
286 producing *Aspergillus carbonarius* in grapevine using molecular markers. *Phytopathology* 98:S64

287 Horie Y (1995) Productivity of ochratoxin A of *Aspergillus carbonarius* in *Aspergillus* section *Nigri*.  
288 *Nippon Kingakukai Kaiho* 36:73-76

289 Larsen TO, Svendsen A, Smedsgaard J (2001) Biochemical characterization of ochratoxin A-producing  
290 strains of the genus *Penicillium*. *Appl Environ Microbiol* 67:3630–3635

291 Mitchell D, Parra R, Aldred D, Magan N (2004) Water and temperature relations of growth and  
292 ochratoxin A production by *Aspergillus carbonarius* strains from grapes in Europe and Israel. *J*  
293 *Appl Microbiol* 97:439-445

294 Mulè G, Susca A, Logrieco A, Stea G, Visconti A (2006) Development of a quantitative real-time PCR  
295 assay for the detection of *Aspergillus carbonarius* in grapes. Int J Food Microbiol 111:S28–S34

296 Nicholson TP, Rudd BAM, Dawson M, Lazarus CM, Simpson TJ, Cox RJ (2001) Design and utility of  
297 oligonucleotide gene probes for fungal polyketide synthases. Chem Biol 8:157-178

298 O'Callaghan J, Caddick MX, Dobson ADW (2003) A polyketide synthase required for ochratoxin A  
299 biosynthesis in *Aspergillus ochraceus*. Microbiol 149:3485–3491

300 O'Callaghan J, Stapleton PC, Dobson ADW (2006) Ochratoxin A biosynthetic genes in *Aspergillus*  
301 *ochraceus* are differentially regulated by pH and nutritional stimuli. Fungal Genet Biol 43:213-221

302 Oliveri C, Torta L, Catara V (2008) A polyphasic approach to the identification of ochratoxin A-  
303 producing black *Aspergillus* isolates from vineyards in Sicily. Int J Food Microbiol 127:147-154

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

307 Perrone G, Susca A, Stea G, Mulé G (2004) PCR assay for identification of *Aspergillus carbonarius*  
308 and *Aspergillus japonicus*. Eur J Plant Pathol 110:641–649

309 Perrone G, Susca A, Cozzi K, Ehrlich J, Varga J, Frisvad JC, Meijer M, Noomin P, Mahakarnchanakul  
310 W, Samson RA (2007) Biodiversity of *Aspergillus* species in some important agricultural products.  
311 Stud Mycol 59:53-66

312 Petzinger E, Ziegler K (2000) Ochratoxin A from a toxicological perspective. J Vet Pharmacol Ther  
313 23:91-98

314 Pietri A, Bertuzzi T, Pallaroni L, Piva G (2001) Occurrence of ochratoxin A in Italian wines. Food  
315 Addit Contam 18:647-654

316 Selma MV, Martínez-Culebras PV, Aznar R (2008) Real-time PCR based procedures for detection and  
317 quantification of *Aspergillus carbonarius* in wine grapes. Int J Food Microbiol 122:126–134

318 Spadaro D, Patharajan S, Karthikeyan M, Lorè A, Garibaldi A, Gullino ML (2009) Molecular  
319 strategies for the identification of *Aspergillus* species in vineyard. J Plant Pathol 91, S4:41

320 Spadaro D, Lorè A, Garibaldi A, Gullino ML (2010a) Occurrence of ochratoxin A before bottling in  
321 DOC and DOCG wines produced in Piedmont (northern Italy). Food Cont 21:1294-1297

322 Spadaro D, Patharajan S, Lorè A, Gullino ML, Garibaldi A (2010b) Effect of pH, water activity and  
323 temperature on the growth and level of ochratoxin A produced by three strains of *Aspergillus*  
324 *carbonarius* isolated from Italian vineyards. Phytopathol Medit 49:65-73

325 Zimmerli B, Dick R (1996) Ochratoxin A in table wine and grape juice: occurrence and risk  
326 assessment. Food Addit Contam 13:655-668

327

328 **Figure legends**

329

330 **Fig. 1.** PCR products obtained from genomic DNA of various *Aspergillus* and *Penicillium* species  
331 using primers AcPKS-F1/ AcPKS-R1. Amplification was observed only for *A. carbonarius* strains and  
332 not for the other species. Lane 1: *A. ellipticus* CBS 707.79; lane 2: *A. tubingensis* CBS 119556; lane 3:  
333 *A. niger* CBS 119557; lane 4: *A. aculeatus* CBS 116.80; lane 5: *A. japonicus* CBS 119560; lane 6: *A.*  
334 *brasiliensis* CBS 121619; Lane 7: *A. ochraceus* CBS 132.52; Lane 8: *P. nordicum* CBS 110769; Lane  
335 9: *P. verrucosum* CBS 226.71E; Lane 10: *A. carbonarius* CBS 127.49; Lane 11: *A. carbonarius* AC06  
336 (Agroinnova culture collection centre); lanes 12 to 18: *A. carbonarius* isolates from Italian vineyards;  
337 Lane M: 100 bp plus molecular weight marker (Gelpilot, Qiagen).

338

339 **Fig. 2.** Ochratoxin A production in fungal mycelium by *A. carbonarius* strain AC06 on two different  
340 media (PDB and YES) incubated at different temperatures. OTA accumulation was determined by  
341 HPLC. Error bars denote the standard error of the mean of three replicates from independent cultures.

342

343 **Fig. 3.** RT-PCR analysis of *AcPks* gene in *A. carbonarius* strain AC06, performed on the cDNA of 9  
344 day-old mycelium grown in two different media (PDB and YES) incubated at different temperatures.  
345 Lanes 1, 2 and 3: mycelium grown on PDB at 10°C, 15°C and 30°C; Lanes 4 and 5: mycelium grown  
346 on YES at 15°C and 30°C; M: 50 bp molecular weight marker (Gelpilot, Qiagen).