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DNA-based characterization of ochratoxin-A-producing and non-producing *Aspergillus carbonarius* strains from grapes

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Abstract

Using molecular methods, a total of 189 strains of black aspergilli, including *Aspergillus carbonarius* and uniseriate species (*Aspergillus aculeatus*, *Aspergillus japonicus*), were studied in order to characterize species responsible for ochratoxin A (OTA) contamination of grapes from Europe and Israel. Sixty-six strains were morphologically identified as belonging to the uniseriate species and 123 as *A. carbonarius*. None of the uniseriate species were able to produce OTA. From the *A. carbonarius* strains, 96.7% were OTA producers (0.1–654.3 μg/g). We characterized 53 strains of *A. carbonarius* from different countries by RAPD and ITS-5.8S rDNA sequencing analysis. Forty-nine strains had a similar RAPD pattern and identical ITS-5.8S rDNA sequences. They produced OTA at different levels. No correlation was observed between the obtained clusters and the OTA production level or origin. Only four strains, morphologically identified as *A. carbonarius*, were unable to produce OTA. These strains showed a different RAPD pattern, and the section of DNA sequenced differed from the sequence of the other 49 strains. These OTA-non-producing strains may represent a new species in the *Aspergillus* section *Nigri*.
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Keywords: *Aspergillus carbonarius*; *Aspergillus japonicus*; *Aspergillus aculeatus*; Black aspergilli; Ochratoxin A; RAPD; ITS-5.8S sequences; Wine grapes

1. Introduction

Next to cereals, wine is considered the second major source of ochratoxin A (OTA) in Europe. Up to now, maximum OTA levels have been established for cereals and dried vine fruits in the European Union [6]. Maximum OTA levels for coffee beans, ground roasted coffee, soluble coffee, wine and other wine and/or grape must-based drinks as well as grape juice and grape juice ingredients in other drinks proposed by the European Commission have been recently approved by the Standing Committee on the Food Chain and Animal Health [7].

Although *Aspergillus ochraceus* and *Penicillium verrucosum* are considered the classical OTA-producing species, they are probably unimportant sources of this mycotoxin in grapes. *P. verrucosum* is usually found in cool temperate regions and has been reported almost exclusively in cereals and cereal products, while *A. ochraceus* is found sporadically in different commodities in warmer and tropical climates [30]. Recently, *A. carbonarius* and other black *Aspergillus* species belonging to the *A. niger* aggregate have been described as a main possible source of OTA contamination in grapes worldwide [8,9,12,15,25,26,31,34]. They have also been reported as sources of OTA in coffee [36].

On the other hand, the taxonomy of black aspergilli (*Aspergillus* section *Nigri*) is not clear and many attempts have been made in order to find accurate criteria for species identification [3]. This section has long been studied by means of morphological and cultural criteria. Some species, such as *A. carbonarius* and uniseriate species (*A. japonicus*, *A. aculeatus*) can be easily recognized. Other species, such as *A. helicothrix*, *A. ellipticus* and *A. heteromorphus* are rare. The *A. niger* aggregate assembles a group of closely related morphospecies in this section. Although speciation at the molecular level has been proposed, no clear morphological

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differences can be observed in this group and species identification remains problematic. A RFLP technique has been proposed as a method for screening possible OTA-producing *A. niger* aggregate strains [5]. Following this technique, at the moment all the OTA-producing isolates whose RFLP pattern is known have been classified as type N, whereas none of the type T strains have been able to produce this mycotoxin.

Only some species in the *Aspergillus* section *Nigri* produce OTA. At present, several members belonging to the *A. niger* aggregate [1] and *A. carbonarius* [19] are considered as clearly ochratoxin A-producing species. Reported percentages of ochratoxigenic isolates belonging to the *A. niger* aggregate are much lower than in *A. carbonarius* [2]. The ability of the uniseriate black *Aspergillus* species to produce OTA has been recently mentioned [8,16], but this fact needs to be confirmed, since at present they are not considered as OTA-producing species [25,28,37].

The objectives of this study were to identify potential ochratoxigenic strains belonging to *A. carbonarius* and uniseriate black *Aspergillus* species from grapes of different origins and to characterize, using molecular methods, representative *A. carbonarius* strains from grapes, which are the main responsible fungi involved in OTA contamination of wine.

2. Materials and methods

2.1. Strains

A total of 189 strains of black aspergilli, including *A. carbonarius* and uniseriate species (*A. aculeatus*, *A. japonicus*), were studied. These strains were supplied by different partners and countries involved in the European project QLK1-CT-2001-01761 (Wine-ochra risk). They were isolated during the years 2001 and 2002. Identification of different strains of black aspergilli was made using macroscopic and microscopic morphological criteria in accordance with appropriate keys [3,22,23,30]. They were preserved at −80 °C in the culture collection of the Faculty of Veterinary Science of Barcelona, Spain.

2.2. DNA extraction

Fungal DNA was extracted as described by Accensi et al. [4]. The strains were inoculated in 1.5 ml Eppendorf tubes containing 500 µl of Sabouraud broth (2% glucose, w/v; 1% peptone, w/v) supplemented with chloramphenicol ($1\ mg\,l^{-1}$), and incubated overnight in an orbital shaker at 300 rpm and 30 °C. Mycelium was recovered after centrifugation and washed with NaCl 0.9% (w/v), frozen in liquid nitrogen and ground to a fine powder with a pipette tip. The powder was incubated for 1 h at 65 °C in 500 µl extraction buffer (50 mM Tris–HCl, 50 mM EDTA, 3% SDS and 1% 2-mercaptoethanol). The lysate was extracted with phenol:chloroform (1:1, v/v), 3 M NaOAc and 1 M NaCl. DNA was recovered by isopropanol precipitation. The pellet was washed with 70% (v/v) ethanol, dried under vacuum and resuspended in TE buffer (Tris–HCl 10 mM, EDTA 1 mM, pH 8). DNA was cleaned with "Geneclean kit II" (BIO 101, Inc., La Jolla, CA), according to the manufacturer's instructions.

2.3. RAPD characterization of A. carbonarius strains

Fifty-three strains that were morphologically identified as *A. carbonarius* were characterized by RAPD. The isolated DNA was used as template DNA for RAPD-PCR. PCRs were performed in a total volume of 25 µl containing 1–10 ng of genomic DNA, 0.5 µM of primer, 0.1 mM dNTPs (Perkin–Elmer, Cerdanyola, Spain) and 1 U of taq DNA polymerase in the buffer provided by the manufacturer (Perkin–Elmer, Cerdanyola, Spain). The primer used was ARI 1 (5′-TGCTTGGCACAGTTGGCTTC-3′) [14].

Amplification reactions were performed in a Perkin–Elmer 2400 DNA thermal cycler which was set to the following conditions: 95 °C, 5 min; 44 cycles consisting of 60 s at 95 °C, 60 s at 36 °C and 4 min at 72 °C. This was followed by a final amplification period of 7 min at 72 °C. At least two separate amplifications were conducted for each isolate. The amplified DNA products were separated by electrophoresis in 2% (w/v) agarose (molecular biology certified agarose, Bio-Rad Laboratories S.A., Barcelona, Spain) in 1× TBE buffer according to Sambrook et al. [33]. Gels were stained in an 1.0 µg/ml ethidium bromide solution and photographed on a gel documentation system (Gel Doc 2000, Bio-Rad Laboratories S.A., Barcelona, Spain). Size marker (100-bp ladder, Bio-Rad Laboratories S.A., Barcelona, Spain) was run on each gel.

The RAPD profiles obtained were used to calculate Dice's coefficient [17] of genetic similarity. A dendrogram was constructed from this coefficient with the UPGMA method [35] of the Bio-Rad Diversity Database Fingerprinting software.

2.4. A. carbonarius ITS-5.8S rDNA sequencing analysis characterization

Fifty-three strains that were morphologically identified as *A. carbonarius* were sequenced. The isolated DNA was used as template DNA. ITS rDNA and 5.8S rDNA were amplified as described by Gené et al. [18], using a Perkin–Elmer 2400 thermal cycler. The primer pairs ITS5 and ITS4 used were described by White et al. [40]. The amplification process consisted of a pre-denaturation step at 94 °C, for 5 min, followed by 35 cycles of denaturation at 95 °C/30 s, annealing at 50 °C/min and extension at 72 °C/min, plus a final extension of 7 min at 72 °C. The molecular masses of the amplified DNA were estimated by comparison with the 100-bp DNA ladder (Bio-Rad Laboratories S.A., Barcelona, Spain) standard lane.

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