International Journal of Food Microbiology 131 (2009) 162–167

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

ITS-based detection and quantification of *Aspergillus ochraceus* and *Aspergillus westerdijkiae* in grapes and green coffee beans by real-time quantitative PCR

Jéssica Gil-Serna [a], Amaia González-Salgado [b], Mª Teresa González-Jaén [b], Covadonga Vázquez [a], Belén Patiño [a,*]

[a] Department of Microbiology III, Faculty of Biology, University Complutense of Madrid, José Antonio Novais 2, E 28040 Madrid, Spain
[b] Department of Genetics, Faculty of Biology, University Complutense of Madrid, José Antonio Novais 2, E 28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 9 June 2008
Received in revised form 12 January 2009
Accepted 9 February 2009

Keywords:
Aspergillus ochraceus
Aspergillus westerdijkiae
Real-time qPCR
OTA
Grapes
Green coffee beans
ITS

ABSTRACT

Aspergillus ochraceus and *A. westerdijkiae* are considered the most important Ochratoxin A (OTA) producing species included in *Aspergillus* section *Circumdati* which contaminate foodstuffs and beverages for human consumption. In this work a real-time quantitative PCR protocol was developed to detect both species using SYBR® Green and primers designed on the basis of the multicopy ITS1 region of the rDNA. The assay had high efficiency (94%) and showed no inhibition by host or fungal DNA other than the target species. The lower detection limit of the target DNA was 2.5 pg/reaction. Accuracy of detection and quantification by qPCR were tested with genomic DNA obtained from green coffee beans and grapes artificially contaminated with spore suspensions of known concentrations. Spore concentrations equal or higher than 10^6 spore/ml could be detected by the assay directly without prior incubation of the samples and a positive relationship was observed between incubation time and qPCR values. The assay developed would allow rapid, specific, accurate and sensitive detection and quantification of *A. ochraceus* and *A. westerdijkiae* to be directly used in a critical point of the food chain, before harvesting green coffee and grape berries, to predict and control fungal growth and OTA production.

1. Introduction

Ochratoxin A (OTA) is a widespread mycotoxin with nephrotoxic, inmunotoxic, genotoxic and teratogenic properties towards several animal species (Pfohl-Leszkowicz and Manderville, 2007) and has been classified by the International Agency for Research on Cancer as a possible human carcinogen (group 2B) (IARC, 1993). The maximum OTA limits allowed in several food and raw agro-products for human consumption are under legal regulation in the European Union (Commission Regulation, 2006). This mycotoxin occurs in various foodstuffs including cereals and derivatives (Rizzo et al., 2002), coffee (Taniwaki et al., 2003), grapes and grape-products (Varga and Kozakiewicz, 2006), dried fruits (Zinedine et al., 2007) and spices (Rizzo et al., 2002).

OTA is a secondary metabolite produced by several fungal species belonging to the *Aspergillus* and *Penicillium* genera. *Aspergillus ochraceus* was the first OTA-producing species described (Van der Merwe et al., 1965) and it is considered an important species contributing to OTA contamination of coffee, grapes and cereals (Taniwaki et al., 2003; Magnoli et al., 2007). Other important OTA-producing species of the Section *Circumdati* are the more recently described *A. westerdijkiae* and *A. steynii* (Frisvad et al., 2004; Samson et al., 2006). Discrimination among these species and from other closely related species is difficult when conventional methods based mainly on morphological features are used and requires considerable expertise. The application of DNA-based techniques permits rapid, sensitive and specific detection, necessary to devise strategies to control or reduce fungal mass and toxin production at early and critical stages of the food chain, and they are replacing traditional methods in many areas related with food analyses (Niessen et al., 2005). Real-time quantitative PCR (qPCR) has solved the limitations of conventional PCR, providing a tool to accurate and sensitive quantification of target DNA. The most common chemistries, DNA-associating dyes (SYBR Green I) or fluorescently labelled sequence-specific oligoprobes (TaqMan® oligoprobes) are being widely used to develop qPCR assays (Mackay et al., 2007). The lower cost of qPCR based on SYBR Green is an advantage of this method for detection and quantification protocols used in routine analyses of commodities, but it may involve a loss of specificity if primers-dimers or nonespecific fragments are present (Kubista et al., 2006). Because of this, additional controls should be done such as analyzing the reaction products with a melting curve (Ririe et al., 1997). The target sequence used to design the primers is also relevant, because it will condition the power of discrimination and the sensitivity of the assay. Several qPCR assays to detect and quantify ochratoxigenic fungi have been developed using as target

* Corresponding author.
E-mail address: belenp@bio.ucm.es (B. Patiño).

0168-1605/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2009.02.008

constitutive genes (Mulè et al., 2006; Morello et al., 2007) or genes involved in toxin biosynthesis (Schmidt et al., 2004; Atoui et al., 2007; Selma et al., 2008). Sensitivity and specificity of qPCR assays are enhanced when multicopy sequences are used to design specific primers (Suarez et al., 2005). However, none of the qPCR protocols reported so far was designed using the multicopy ITS regions to detect ochratoxigenic fungi.

The applicability of the assay to detect fungi in natural samples need special consideration since food matrices are usually very complex (Hanna et al., 2005) and some of the matrix-associated compounds can inhibit qPCR or reduce its efficiency, such as phenolic compounds which may cause problems in real-time reaction by binding or denaturing the polymerase (Wilson, 1997).

Early detection of OTA-producing species is critical to prevent mycotoxin entering the food chain (Dao et al., 2005) and OTA concentration can be correlated with the levels of ochratoxigenic fungus detected on naturally contaminated samples (Lund and Frisvad, 2003). Hence, identification and quantification of *A. ochraceus* and *A. westerdijkiae* in raw products could predict potential risk of OTA contamination of foodstuffs.

The aim of the present work was to develop a sensitive and specific assay not inhibited by matrix effects to detect and quantify *A. ochraceus* and *A. westerdijkiae* in green coffee beans and grapes. An efficient protocol for extraction of fungal DNA from foodstuffs was developed and the effect of exogenous DNA on the qPCR efficiency was also evaluated.

2. Materials and methods

2.1. Organisms, media and culture conditions

All the isolates used in this study are given in Table 1. Fungal strains were maintained by regular subculturing on Potato Dextrose Agar (PDA) (Pronadisa, Madrid, Spain) at 25 + 1 °C for 4–5 days and then stored as spore suspension in 15% glycerol at −80 °C. Fungal strains were cultured for DNA extraction in Erlenmeyer flasks containing 20 ml of Sabouraud Broth (Pronadisa, Madrid, Spain) and incubated at 28 + 1 °C in an orbital shaker (140 rpm) for 3 days.

2.2. DNA extraction

DNeasy Plant Mini Kit (Qiagen, Valencia, Spain) was used according to manufacturer's instructions starting from 20 mg of filtered mycelium frozen with liquid nitrogen and grinded using a mortar and pestle. This protocol was also used for DNA extraction from green coffee beans. The yield of the method was evaluated in five independent extractions of *A. ochraceus* CECT 2092 and *A. westerdijkiae* ALD and ALF. In the case of DNA extraction from grapes, 0.33% Polyvinylpyrrolidone (PVP) (Sygma-Aldrich, Steinheim, Germany) was added to AP1 and AP2 buffers. In all cases, purified DNA was eluted from the DNeasy spin column using elution buffer (TE) and optimal results were obtained by eluting twice (2×75 µl). DNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA).

2.3. Primer design

Two specific primers to *A. ochraceus* and *A. westerdijkiae*, OCRAQ1 and OCRAQ2 (5'GCACAGCGC TCGCCG 3' and 5'CTGATTGCGATACAATCG 3' respectively) were designed on the basis of sequence alignments of the ITS1 region of several strains from different origins and other related species and genera obtained in our laboratory or retrieved from data bases.

A universal pair of primers based in 5.8 S region, 5.8 S1 (5'CGG-CATCGATGAA GAACGC 3') and 5.8 S2 (5'CAATGTGCGTTCAAAGACTCG 3'), was designed following the same approach indicated above to test amplification in samples where neither *A. ochraceus* nor *A. westerdijkiae* DNA were present.

Table 1
Fungal strains used in this study to validate the quantitative PCR assay designed.

Strain	Species	Source	Amplification with OCRAQ1/OCRAQ2
178[a]	*Aspergillus carbonarius*	Grapes, Spain	–
242[a]*	*Aspergillus carbonarius*	Grapes, Spain	–
325[a]	*Aspergillus carbonarius*	Grapes, Spain	–
CBS 102.14	*Aspergillus elegans*		–
CBS 310.80	*Aspergillus elegans*		–
CBS 614.78	*Aspergillus elegans*		–
IMI 345568	*Aspergillus elegans*		–
ITEM 4592	*Aspergillus flavus*	Wheat, France	–
M12hip10[a]*	*Aspergillus flavus*	Barley, Spain	–
ITEM 4685	*Aspergillus japonicus*		–
B.Me.A26	*Aspergillus niger*	Grapes, Spain	–
CECT 2091	*Aspergillus niger*		–
ALH[b]	*Aspergillus ochraceus*		+
ALM[b]	*Aspergillus ochraceus*		+
CBS 108.08	*Aspergillus ochraceus*		+
CBS 624.78	*Aspergillus ochraceus*		+
CECT 2092*	*Aspergillus ochraceus*		+
CECT 2093	*Aspergillus ochraceus*		+
CECT 2969	*Aspergillus ochraceus*		+
CECT 2970	*Aspergillus ochraceus*		+
Cab5dch6	*Aspergillus parasiticus*		–
CBS 112812	*Aspergillus steynii*	Coffee, India	–
CBS 112814	*Aspergillus steynii*	Coffee, India	–
CBS 121991	*Aspergillus steynii*	Coffee, Thailand	–
CBS 121993	*Aspergillus steynii*	Coffee, Thailand	–
Bo75*	*Aspergillus tubingensis*	Grapes, Spain	–
T.TT.A2	*Aspergillus tubingensis*	Grapes, Spain	–
ALB[b]	*Aspergillus westerdijkiae*		+
ALD[b]*	*Aspergillus westerdijkiae*		+
ALF[b]*	*Aspergillus westerdijkiae*		+
ALG[b]	*Aspergillus westerdijkiae*		+
CBS 112803	*Aspergillus westerdijkiae*	Sorghum, South Africa	+
CBS 112791	*Aspergillus westerdijkiae*		+
CBS 121983	*Aspergillus westerdijkiae*	Coffee, Thailand	+
CBS 121984	*Aspergillus westerdijkiae*	Coffee, Thailand	+
CBS 121986	*Aspergillus westerdijkiae*	Coffee, Thailand	+
F4094*	*Fusarium thapsinum*	Laboratory cross	–
FvMM1-2	*Fusarium verticillioides*	Maize, Spain	–
CECT 2270*	*Penicillium corylophilum*		–
CYC 20012	*Botrytis cinerea*	Grapes, Spain	–
CYC 20013	*Botrytis cinerea*	Grapes, Spain	–
IMI 311661	*Colletotrichum coffeanum*	Coffee, Tanzania	–
CECT 10590	*Pichia anomala*	Grape Juice, Spain	–
CECT 10113	*Pichia membranifaciens*	Grapes, Spain	–
Pv 1	*Plamopara viticola*	Grapes, Spain	–
CECT 1172	*Saccharomyces cerevisiae*	Orange juice, Spain	–
CECT 10676	*Torulaspora delbrueckii*	Grapes, France	–
Un 1	*Uncinula necator*	Grapes, Spain	–

Last nine strains corresponding to species that cause common fungal grapes or green coffee diseases and yeast that could be present as usual flora.
CBS: Centralbureau voor Schimmel Cultures (The Netherlands).
CECT: Spanish Type Culture Collection (Spain).
CYC: Complutense Yeast Collection (Spain).
ITEM: Institute of Sciences of Food Production Culture Collection (Italy).
IMI: CABI Genetic Resource Collection (United Kingdom).
[a] Strain supplied by Dr. V. Sanchis (University of Lleida, Spain).
[b] Strains supplied by Dr. L. Niessen (Technische University of München, Germany).
* Fungal strains used to test the specificity of the qPCR assay.

2.4. Conventional PCR amplification

Specificity of the pair OCRAQ1/OCRAQ2 was tested by conventional PCR in a wide range of isolates shown in Table 1. The assays were performed in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). Amplification reactions were carried out in volumes of 25 µL containing 2 µL (5–50 ng) of template DNA, 1 µL of each primer (20 µM), 2.5 µL of 10× PCR buffer, 1 µL of $MgCl_2$ (50 mM), 0.2 µL of dNTPs (100 mM) and 0.15 µL of Taq DNA polymerase (5 U/µL) supplied by the manufacturer (Biotools, Madrid, Spain).

All genomic DNAs were tested for suitability for PCR amplification using universal primers ITS1 and ITS4 (White et al., 1990) and the