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Predicting the growth/no-growth boundary and ochratoxin A production by *Aspergillus carbonarius* in pistachio nuts

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ABSTRACT

Black aspergilli are the main fungal contaminants in pistachio nuts. Ochratoxin A (OTA) production has been repeatedly reported in *Aspergillus* section *Nigri*; OTA has been occasionally detected in pistachio nuts in high concentrations.

The aim of this study was to develop suitable validated models to predict the growth and OTA production boundaries by an *Aspergillus carbonarius* isolated from pistachios as a function of moisture content and storage temperature of pistachios. A full factorial design was used: the moisture content levels assayed were 12.5%, 17.9%, 24.0%, 29.5% and 34.8% and the incubation temperatures were 10, 15, 20, 25, 30, 37 and 42 °C.

A probability model was built to predict the growth of the strain under the assayed conditions, which proved to be concordant in 73–91% of the cases with the observed probabilities in the validation test specifically chosen for limiting growth conditions. Similarly, the probability for the presence of OTA was correctly predicted in 90% of the cases. OTA accumulation was mainly a function of the temperature of storage, with a sharp increase at <15–20 °C; this value was very different from 30 to 35 °C, which was optimum for growth. Increasing OTA levels were found with increasing moisture content in the pistachio nuts. The impact of these results in the implementation of HACCP plans in Western Asian countries, is discussed.

Probability models were applied in this work to mycotoxin accumulation for the first time, however, further research is required in the kinetics of mycotoxins accumulation to develop proper empirical models.

1. Introduction

Members of the *Aspergillus* spp., amongst many other toxigenic fungi, have through time been found to have a strong ecological link with human food supplies (Pitt, 2000). They are often associated with food and animal feed during drying and storage, but may also occur as plant pathogens. Black aspergilli—*Aspergillus* classified into the section *Nigri* by Gams et al. (1985), formerly "*A. niger* species group" by Raper and Fennell (1965)—, have dark colonies, often black, with uniseriate or biseriate conidiophores. They have been isolated from a wide variety of food and are distributed worldwide (animal feed, cereals, cocoa, coffee, dried fruits, dried grapes, fruits, garlic, olives, onions, pulses) and are considered as common fungi causing food spoilage and biodeterioration of other materials. They have been recently associated with the presence of ochratoxin A (OTA) in grapes and their derivatives (Battilani et al., 2003). The main species involved in OTA contamination is *Aspergillus carbonarius* and a low percentage of isolates of the closely related species in *A. niger* aggregate (Belli et al., 2004).

Several studies have reported the incidence of black aspergilli in pistachios and other tree nuts. *A. niger* was found in 42% of nut samples including pistachios, almonds, walnuts and brazil nuts (Bayman et al., 2002). The contamination of pistachios is of growing concern in production regions such as Iran, Turkey and the USA. In Iran, *A. niger* was the most common *Aspergillus* species isolated from pistachio nuts (not necessarily early split ones) (Doster and Michailides, 1994).

Aflatoxins have often been found in pistachio samples, and EU legislation includes these nuts with a maximum permissive level of $4\,ng\,g^{-1}$. OTA content has been, however, rarely analysed in nut samples, and the responsible fungal species have not been reported. In a survey on 23 samples of pistachio nuts from several different brands, OTA was detected at a high level in one sample (Food Standards Agency and Contaminants Division, 2002).

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The effects of water activity and temperature on growth and OTA production by *A. carbonarius* isolated from grapes have been widely analysed in both grape and synthetic media (Belli et al., 2005, 2007; Marín et al., 2006), and some models generated. Models allow microbial responses to be predicted at conditions that have not been specifically studied. There are no reports describing the effects of moisture content and temperature specifically on the growth and OTA production of black aspergilli in pistachios. In order to improve the quality and safety of food, there is a need for tools allowing the prediction of toxin contamination.

The aim of this study was to develop suitable validated models to predict the growth and OTA production boundaries by an *A. carbonarius* isolate as a function of moisture content and storage temperature of pistachios.

2. Methods

2.1. Pistachio nuts

Pistachios were purchased from a wholesaler in Lleida, Catalunya, Spain, on February 2007. Origin was Teheran, Iran, grade: 5 star; type: round; packing date: October 28, 2006; expiration date: October 28, 2008.

2.2. Fungal isolate

Direct plating of 100 surface-disinfected pistachio nuts from the above batch on MEA showed that 100% of the nuts were infected by black aspergilli; the isolates belonged in 99% of the cases to the *A. niger* aggregate, except for one isolate of *A. carbonarius*, which was used in this study. Of the *A. niger* aggregate isolates, 5% were OTA positive, while the single *A. carbonarius* isolate was OTA-positive, and produced much higher amounts of OTA than those from the former group. The OTA-producing capacity of the isolates was checked using the method of Bragulat et al. (2001).

2.3. Experimental design

A full factorial design was used in which two factors were assayed: moisture content and temperature. The moisture content levels assayed were 12.5%, 17.9%, 24.0%, 29.5% and 34.8% and the incubation temperatures were 10, 15, 20, 25, 30, 37 and 42 °C. Ten replicates for each treatment were used.

2.4. Pistachios rehydration, inoculation and incubation

Thirty 1-l bottles were filled with 250 g of hulled pistachios and autoclaved at 121 °C for 20 min; due to the low initial water content, no softening was observed in the nuts due to autoclaving. From the 30 bottles, every six were adjusted to a different moisture content level. An adsorption isotherm was initially determined and used (Fig. 1). According to this, 24.32, 42.99, 63.05, 84.88 and 109.04 ml water g^{-1} were required to achieve about 10%, 15%, 20%, 25% and 30% moisture content, respectively. Then they were stored in the fridge (+7 °C) for 72 h with periodic mixing. Both final moisture content and water activity were quantified on the treatment and replicate samples.

Pistachios were decanted from the bottles into Petri plates (10 Petri plates from each bottle) to form a single layer. Petri plates were inoculated centrally with a loop of *A. carbonarius* spore suspension (10^6 spores ml^{-1}) prepared from a pure culture grown on malt extract agar for 7 days.

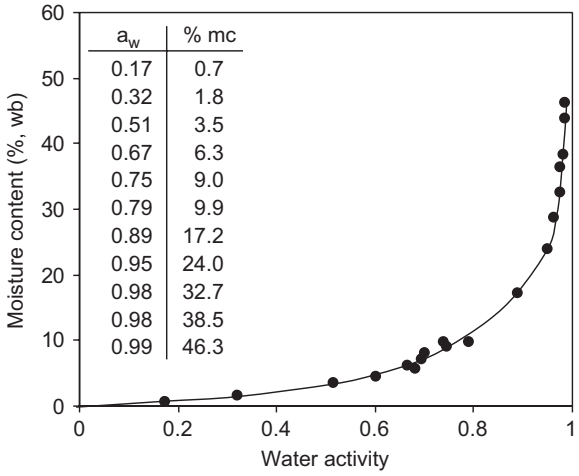

Fig. 1. Experimental sorption curve of pistachio nuts.

Table 1
Timing of the OTA analysis (incubation days)

Temperature (°C)	Moisture content				
	12.6%	17.9%	24.0%	29.5%	34.8%
10	35, 90 d	35, 90 d	35, 90 d	7, 35 d	7, 35 d
15	35, 90 d	18, 35 d	18, 35 d	7, 18 d	7, 18 d
20	35, 90 d	18, 35 d	18, 35 d	7, 18 d	7, 18 d
25	35, 90 d	18, 35 d	18, 35 d	7, 18 d	7, 18 d
30	35, 90 d	18, 35 d	18, 35 d	7, 18 d	7, 18 d
37	35, 90 d	18, 35 d	18, 35 d	7, 18 d	7, 18 d
42	35, 90 d	35, 90 d	35, 90 d	18, 35 d	18, 35 d

Ten plates with the same moisture content were taken at random and placed in a plastic container with two beakers filled with specific glycerol + water solutions to maintain the same relative humidity in the atmosphere in the container as the treatment plates. Plastic containers (30 in total) were incubated at seven different temperature levels (10, 15, 20, 25, 30, 37 and 42 °C).

2.5. Growth assessment

Growth was daily observed for between 17 days and 3 months, depending on the suitability of the tested conditions for growth. The observations were carried out with the aid of a binocular magnifier. The first day when growth was observed in each plate was recorded.

2.6. OTA analysis

Petri plates were removed from incubation following the sequence presented in Table 1.

Pistachio nuts were analysed for OTA using immunoaffinity clean-up columns. The protocol suggested by the manufacturer (Ochraprep, Rhône Diagnostics Technologies, Glasgow, UK) for cereals was used. A mean recovery rate of 86% was obtained using this method with analysed spiked samples between 5 and 20 ng g^{-1}. The content of each plate was weighed and ground. Each ground sample was extracted (1+4 w/v) with 60% acetonitrile in water by blending for 20 min. Extracts were filtered and 4 ml were diluted with 44 ml phosphate-buffered saline pH 7.4